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THE UNIVERSITY OF ALBERTA

THE ANAEROBIC BIODEGRADATION OF 4-CHLOROPHENOL AND 2,4-DICHLOROPHENOL

BY

ZOHRA FATIMA ALI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

IN

ENVIRONMENTAL SCIENCE
DEPARTMENT OF CIVIL ENGINEERING

EDMONTON, ALBERTA
SPRING, 1991

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AND 2,4-DICHLOROPHENOL

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Dedicated to:

My parents,
Dr. S. M. Farouq & Mrs. Razia Ali

My brother,
Yousuf

My sister,
Fozia

Abstract

There is an increasing interest in the anaerobic biodegradation of chlorophenols because of their persistence in the environment. Pollution sources of chlorophenols are primarily industrial wastes, sewage treatment plants, and pesticides. This research concentrated on the formation of intermediates and stoichiometry involved in 4-chlorophenol biodegradation using sewage sludge as the inoculum. Work was also done with 2-chlorophenol and 2,4-dichlorophenol.

Initial analysis of radioactive 4-chlorophenol and 2,4-dichlorophenol-containing cultures indicated that nearly 50% of 4-chlorophenol and more than 70% of 2,4-dichlorophenol adsorbed to sewage particles. Sterile and non-sterile sludges adsorbed 60% of the 2,4-dichlorophenol in cultures that were centrifuged so that the 2,4-dichlorophenol concentration in the supernatant could be analyzed by gas chromatography. Sterile and non-sterile cultures that were sampled directly from the cultures adsorbed 52% and 65%, respectively.

Dilution experiments intended to dilute toxic substances that might inhibit 2-chlorophenol and 4-chlorophenol degradation achieved the opposite effect. The 4-chlorophenol concentration did not change in 34 days, and thus, the cultures were terminated. However, the 2-chlorophenol cultures displayed increased lag periods with increasing dilutions as well as decreased degradation rates. All dilutions, with initial concentrations of 25 mg/L of 2-chlorophenol produced phenol, which peaked when the concentration of 2-chlorophenol was below 3 mg/L.

The dechlorination rate of 2-chlorophenol was higher with

fresh sludge from the City of Edmonton wastewater treatment plant rather than laboratory digestors using phenol as sole carbon sources. The dechlorination rate with fresh sludge was 11 mg/L/day.

The degradation rate of 4-chlorophenol varied considerably, which was a reflection of the difficulty in obtaining a homogeneous, active population. The degradation rate varied from 0.3 mg/L/day to 6.9 mg/L/day. Cultures that received 4-chlorophenol very soon after the point at which the first amount was depleted, showed much higher degradation rates.

Mineralization of both 4-chlorophenol and 2,4-dichlorophenol was proven by the production of radioactive carbon dioxide and methane in the culture headspace. Analysis of the supernatant of active 4-chlorophenol cultures resulted in the identification of only acetate as an intermediate.

Determining the stoichiometry of phenol and 4-chlorophenol degradation was not possible because of the high proportion of radioactivity that remained associated with the liquids and solids of the cultures.

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List of Abbreviations

COD = chemical oxygen demand

-COOH = carboxyl group

cm = centimeters

CB = chlorobenzoate

CP = chlorophenol

°C = degrees centigrade

diCB = dichlorobenzoate

diCP = dichlorophenol

2,4-D = 2,4-dichlorophenoxyacetic acid

DPM = disintegrations per minute

ft = feet

GPC = gas proportional counter

$\Delta G^{\circ\prime}$ = Gibbs free energy

g = grams

GAC = granular activated carbon

h = hours

ht = height

H = Henry's law constant

HCB = hexachlorobenzene

HV = high voltage

-OH = hydroxyl group

ID = inner diameter

kg = kilograms

K_G = gas-phase mass transfer coefficient

kJ = kilojoules

K_L = liquid-phase mass transfer coefficient

K_{oc} = soil/sediment partition coefficient

K_{ow} = octanol-water partition coefficient

Km = the concentration of substrate at half the maximum rate of reaction (1/2 Vmax)

Kp' = adsorption coefficient

L = liter

LSC = liquid scintillation counter

MATC = maximum acceptable toxicant concentration

mg = milligrams

m/h = meters per hour

m/s = meters per second

mm = millimeters

mM = millimoles per liter

mos = months

mV = millivolts

min = minutes

ng = nanograms

N = normality (equivalents per liter)

pentaCP = pentachlorophenol

PCB = polychlorinated biphenyl

pKa = acid dissociation constant

rpm = revolutions per minute

silvex = 2-(2,4,5-trichlorophenoxy)propionic acid

spd = speed

STP = standard temperature and pressure

tetraCP = tetrachlorophenol

TLC = thin layer chromatography
TSS = total suspended solids
TCA = tricarboxylic acid
TCB = trichlorobenzene
triCP = trichlorophenol
2,4,5-T = 2,4,5-trichlorophenoxyacetic acid
UV = ultraviolet
Vmax = the maximum rate of reaction
VSS = volatile suspended solids
%w/v = percent weight by volume
µg = micrograms
µL = microliters
µ = microns

1. Introduction

Chlorophenols are a continuous source of pollution in the environment. Their persistence has prompted many researchers to study the biodegradation of chlorinated phenols, both in aerobic and anaerobic environments. The ease with which these compounds are metabolized as well as intermediates formed during metabolism are of particular interest to further understand the fates of these compounds. This thesis concentrates on the anaerobic biodegradation of 4-chlorophenol (4-CP) using anaerobic sewage sludge as the inoculum.

2. Sources of chlorophenols in the environment

2.1. Industrial sources

Chlorophenols are commonly found in industrial waste. This includes wastes from wood-processing plants, wood-preservation plants, kraft pulp mills, and sewage treatment plants (NRCC, 1982).

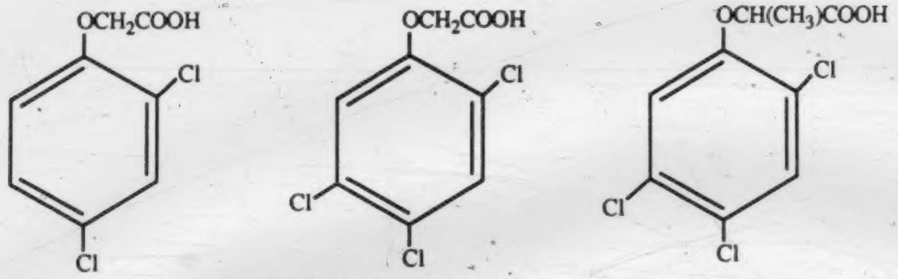
The major use of chlorophenols is in wood preservation. Annual use of chlorophenols in Canada could be as high as 6.0×10^6 kilograms. Thus, there is considerable scope for environmental contamination if chlorophenols persist in the environment (NRCC, 1982; Dewalle et al., 1982).

Some of the monochlorophenols and dichlorophenols have other specific uses. 4-CP is used as an alcohol denaturant and as a solvent to refine mineral oils (Krijgsheld and van der Gen, 1986). 2,4-Dichlorophenol (2,4-diCP) is used as one of the starting components in the production of the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D is widely used to control broadleaf weeds and hardwood trees. It is also used to brush on crop land, in forests, and on lawns. The alkali salts of 2,4-diCP are utilized as germicides and antiseptics (Krijgsheld and van der Gen, 1986).

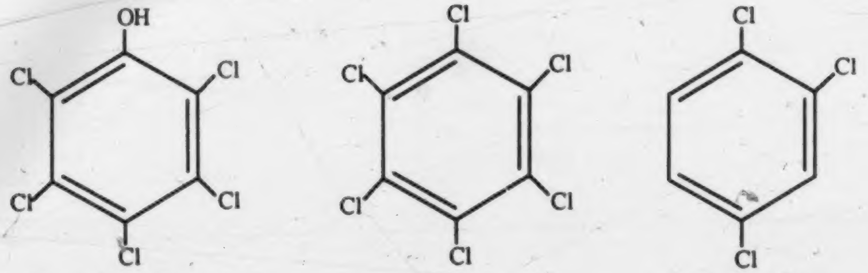
Pentachlorophenol (pentaCP) is a pesticide used to prevent mildew formation on textiles and to prevent mold and slime formation in heat exchange systems (Ingols et al., 1966). Both penta- and 2,3,4,6-tetrachlorophenol (2,3,4,6-tetraCP) are used as fungicides on freshly cut wood, and therefore are found in sawdust and wood shavings (Alexander, 1974).

2.2. Products of pesticide degradation

A major source of chlorophenols is the degradation of chlorinated hydrocarbons that are used as pesticides. Figure 1 illustrates the structures of some of these pesticides. The primary group of pesticides that yield chlorophenols as degradation products are the phenoxyacetates. These include 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Munnecke et al., 1982). The phenoxyacetates are the most commonly used pesticides in the world (Zepp et al., 1975). They are generally used to control broadleaf weeds that infest grains such as wheat, oats, and corn (CAST, 1978). Also, they are used in forests, on grazing land, and in water bodies such as lakes and ponds to kill floating weeds (CAST, 1978). Thus, these pesticides are directly applied and intended to persist for maximum effect. In addition to direct application, phenoxyacetates can enter the environment through chemical spills, through leakage from storage areas possibly filtering into groundwater, and through discharges from water and wastewater


OCH$_2$COOH
Cl
Cl
OCH$_2$COOH
Cl
Cl
Cl
OCH(CH$_3$)COOH
Cl
Cl
Cl

2,4-Dichlorophenoxyacetic acid (2,4-D)   2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)   Silvex


OH
Cl
Cl
Cl
Cl
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Cl
Cl
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Cl
Cl
Cl
Cl
Cl
Cl

Pentachlorophenol (PentaCP)   Hexachlorobenzene   1,2,4-Trichlorobenzene (1,2,4-TCB)

Figure 1. Chemical Structure of Some Chlorinated Pesticides
(Adapted from Merck Index, 1983)

treatment plants into natural water bodies. In the last case, however, it is presumed that only acceptable levels of pesticides are being discharged (Young, 1984).

The most intensively investigated phenoxyacetate has been 2,4-D. Most of the research conducted has focused on the aerobic biodegradation of 2,4-D, always resulting in 2,4-diCP as one of the metabolites. Loos et al. (1967) studied the aerobic biodegradation of 2,4-D by an Arthrobacter species. A soluble fraction of disrupted cells (from which whole cells and fragments of cells were removed) produced 2,4-diCP upon incubation with 2,4-D as the substrate. Evans et al. (1971) isolated a Pseudomonas sp. from a soil enrichment and noted the production of 2,4-diCP when the culture was incubated with 2,4-D as the substrate. Alexander (1974) noted that at a concentration of 50 mg/L, under aerobic conditions, 2,4-D yielded 2,4-diCP when incubated in soil or water. However, the amount of 2,4-diCP formed was less than 0.3 mg/L.

In addition to biological degradation, 2,4-D is also subject to non-biological decomposition. Zepp et al. (1975) irradiated a water solution containing less than 1 mg/L of the 2,4-D ester and found 2,4-diCP as one of the major products of photodecomposition. The irradiation was carried out using polychromatic light to obtain the wavelengths found in sunlight. However, when using sterile river water (passed through a 0.22 µm filter prior to use) rather than distilled water, they found a greater amount of 2,4-diCP had formed, with the photodecomposition rate in river water twice that in distilled water, suggesting that other factors in natural bodies of water influence photodecomposition.

The pesticide 2,4,5-T has been found to be more resistant to biodegradation than 2,4-D (Rochkind-Dubinsky et al., 1987; Alexander and Aleem, 1961). Rosenberg and Alexander (1980a) studied the biodegradation of 2,4,5-T in enriched soil, noting that 2,4,5-trichlorophenol (2,4,5-triCP) was one of the products. When clay was initially incubated with glucose and benzoate, a greater yield of trichlorophenol was seen, compared to clay that was not amended. Rosenberg and Alexander (1980b) also investigated 2,4,5-T biodegradation using sewage or tropical soil as the inoculum. The degradation in the soil was quite slow, with a lag time of two and a half months. By four months, 20% to 80% of the 2,4,5-T was removed. Forty-one bacterial colonies were isolated from sewage or soil samples. Some of these isolates produced 2,4,5-triCP as well as mono- and dichlorophenols as products. McCall et al. (1981) investigated the aerobic degradation of 2,4,5-T in soil without light. Using thin layer chromatography (TLC) 2,4,5-triCP was found to be a degradation product. The degradation curve was sigmoidal meaning that the degradation rate increased until 20% of the 2,4,5-T was left, then the rate decreased.

Several other chlorinated pesticides have been shown to produce chlorophenols as degradation products. Sackmauerova-Veningerova et al. (1981) studied the biodegradation of hexachlorobenzene (HCB) in poultry liver and meat during an experiment in which HCB was added to feed. Some of the degradation products were 2,4-diCP, 2,4,6-trichlorophenol (2,4,6-triCP), and pentaCP. Marinucci and Bartha (1979) incubated trichlorobenzene (TCB) continuously under anaerobic conditions or alternately with oxygen. In both aerobic and anaerobic conditions, 1,2,4-TCB formed 2,4-, 2,5-, and 3,4-diCP with 2,4-diCP being the most often identified product. Ou and Sikka (1977) investigated the biodegradation of 2-(2,4,5-trichlorophenoxy)propionic acid (silvex) using enriched pond water as the inoculum. The enriched pond water was pond water that was incubated with 3 g/L silvex at a temperature of 23°C and a pH of 7.3. A colony consisting of both a *Pseudomonas* species and an *Achromobacter* species degraded silvex, forming 2,4,5-triCP as one of the products. Interestingly, colonies consisting of either the *Pseudomonas* species or the *Achromobacter* species alone, could not degrade silvex, indicating the need for interaction between the two organisms.

2.3. By-product reactions in manufacture

A number of different chlorophenols can be formed during the synthesis of 2,4,6-triCP, pentaCP, and 2,4-diCP (Hutzinger and Veerkamp, 1981; Krijgsheld and van der Gen, 1986). Hutzinger and Veerkamp (1981) noted that the production of 2,4,6-triCP could result in chlorophenols containing several chlorinated substituents, in addition to 2,4,6-triCP. In addition, they found that the production of pentaCP resulted in the formation of tetrachlorophenols as by-products.

Krijgsheld and van der Gen (1986) noted that 2,6-diCP and 2,4,6-triCP are formed during the production of 2,4-diCP in which phenol is chlorinated. If 4-CP is chlorinated, the resultant 2,4-diCP is purer. If 2-CP is chlorinated, one of the additional products formed, other than the desired 2,4-diCP, is 2,6-diCP.

Cochrane et al. (1983) investigated ten technical samples of 2,4-diCP and found an average of 92.24% 2,4-diCP actually present in the samples. In addition to 2,4-diCP, they also found by-products such as 4.48% 2,6-diCP, 1.24% 2,4,6-triCP, 1.23% 2-CP, and 0.41% 4-CP. They suggested that the by-products were probably the result of under- and over-chlorination. As well as 2,4-diCP, Cochrane et al. (1983) also studied thirteen technical samples of 2,4-D, finding 2,4-diCP present at 0.19% as the major impurity. Lesser amounts of 2-CP, 4-CP, 2,6-diCP, and 2,4,6-triCP were also identified as by-products.

2.4. Chlorophenols from the chlorination of water and wastewater

In water and wastewater treatment, water is chlorinated as a disinfection process. Sewage, in particular, contains a variety of organics, including phenol, so many chlorination products may form as a result of disinfection of wastewaters.

Onodera et al. (1984) found that a number of chlorophenols were formed after reacting phenol with hypochlorite in dilute aqueous solution at 20°C. The chlorophenols formed were 2-CP, 4-CP, 2,4-diCP, and 2,4,6-triCP.

Murphy et al. (1975) reacted phenol and free chlorine under conditions of wastewater disinfection. Their study found that ring structures containing electron activating components such as -OH could be chlorinated under wastewater disinfection conditions. Further, they found the presence of 2-CP, 2,6-diCP, and 2,4,6-triCP after reacting the chlorinated water with phenol.

Similarly, Voudrias et al. (1985) were also interested in reaction products formed between phenol and free chlorine under conditions encountered in drinking water treatment. However, they were also interested in the role played by granular activated carbon (GAC). They found that mono-, di-, and trichlorophenols were formed when phenol and free chlorine reacted in solution. As well, chlorophenols were identified within the GAC pore solution. That is, they were within the matrix of the GAC. In addition, identical reaction products were formed within the GAC pore solution. Thus, identical reaction products were formed outside of and within the GAC when a high phenol concentration was applied. However, it was noted that less chlorinated products were formed when the phenol was preadsorbed to the GAC surface, possibly caused by the hindrance of the pores to the chlorine.

Voudrias et al. (1985) investigated the effect of pH on the reaction, and found the same products both at pH 3.1 and at pH 6.0., although, at a high pH, chlorinated phenol derivatives were not detected. 4-CP was reacted with chlorine, and 20% of the 4-CP formed chlorohydroxybiphenyls, or hydroxylated PCB's (polychlorinated biphenyls). The effect of the activated carbon used did not appear to be significant, because the same reaction products were formed with different carbons.

Glaze and Henderson (1975) bubbled chlorine gas into stirring secondary municipal effluent with a contact time of one hour and a pH of 2 to 3. The presence of tetraCP was confirmed by comparison to a reference.

3. Health concerns over chlorophenols in the environment

3.1. Occurrence

Concern over the presence of chlorophenols in drinking water supplies as well as in food ingested by man, has prompted the investigation of food products and waterways for the presence of chlorophenols. Bjerke et al. (1972) fed cows phenoxyherbicides by adding 2,4-D or 2,4,5-T to feed at a concentration ranging from 10 to 1000 mg/L. No residues of 2,4-diCP greater than 0.05 mg/L were identified in the milk or cream of cows fed a concentration of 300 mg/L or lower. Even at the 1000 mg/L level, an average of less than 0.05 mg/L 2,4-diCP was detected in the milk. Cows fed 1000 mg/L 2,4,5-T had 0.23 mg/L 3,4,5-triCP in milk, and 0.19 mg/L in cream. Also, when the chemicals were no longer included in the feed, there was a rapid loss of the chemicals from the milk.

Several studies have been conducted to determine the levels of chlorophenols in natural bodies of water. Wegman and Hofstee (1979) analyzed surface samples in the Rhine river and found that 2,6-diCP, 2,4,6-triCP, and pentaCP were most frequently identified. Pentachlorophenol was found 100% of the time and had a maximum concentration of 11 µg/L, the highest among the chlorophenols. Wegman and van den Broek (1983) again studied chlorophenol levels in the Netherlands and found 2,4,6-triCP and pentaCP occurring the most frequently. Morgade et al. (1980) analyzed both tap water and well water for the presence of chlorophenols. An average concentration of 98 ng/L pentaCP was detected in the tap water, and 44 ng/L pentaCP was found in well water. It was suggested that this two-fold difference between tap and well water may be a reflection of the chlorination process used to disinfect water in treatment plants. Also, industrial wastes entering the waterways are more likely to end up in treatment plants rather than in well water which is more protected from industrial pollution.

3.2. Human health

Generally, the oral route is the primary route through which the chlorophenols may enter the body. This is especially true for contaminated drinking water supplies and for tainted fish and meat that are ingested. Workers that come into direct contact with these compounds could also experience significant exposure by the dermal route depending on the degree of protection offered the worker. Because most of the toxicity studies have been conducted on rodents, the results must be carefully extrapolated to translate any findings to reach conclusions concerning human toxicity.

Chlorophenols generally cause skin irritations. In addition, pentaCP dusts and sprays result in painful irritations of the eyes and respiratory tract. Symptoms of intoxication by pentaCP include appetite loss, sweating, respiratory problems, gastrointestinal upset, and, in severe cases, coma (Clayton and Clayton, 1981).

As well as producing a potential health hazard, the presence of chlorophenols in water is an aesthetic problem. Burttschell et al. (1959) determined taste and odor thresholds for several chlorophenols. They found that the chlorophenol taste was not produced at a pH of less than 7.0. Both 4-CP, and 2,4,6-triCP had high taste (> 1000 µg/L) and odor thresholds (4-CP: 250 µg/L, 2,4,6-triCP: > 1000 µg/L), thus, they were considered negligible contributors to taste and odor problems. However, 2-CP, 2,4-diCP, and 2,4,6-triCP had taste thresholds of 4, 8, and 2 µg/L, respectively. Their corresponding odor thresholds were 2, 2, and 3 µg/L, respectively.

Chlorophenols have been detected in human body fluids as well as in human fatty tissue. Edgerton et al. (1980) confirmed the presence of chlorophenols in human urine. An average of 34 µg/L 2,4- and/or 2,5-diCP was detected in 12 samples, and an average of 15 µg/L pentaCP was also detected. In addition, Lores et al. (1981) confirmed the presence of chlorophenols in human urine at the µg/L level. Hargesheimer and Coutts (1983) analyzed more than four hundred urine samples and found the average 2,4,5-triCP and pentaCP concentrations were less than 6 µg/L.

Bevenue et al. (1968) analyzed the blood of people that were frequently and directly exposed to pentaCP in their working environment. In most of the cases studied, the majority of pentaCP detected was found in the blood plasma, whereas, the cell fraction contained only 1% after washing with saline solution. Morgade et al. (1980) found pentaCP present in blood serum ranging from 10 to 98 µg/L for people that drank tap water (average 98 ng/L) regularly, and a range of 11 to 120 µg/L for people that drank well water (average 44 ng/L) regularly.

Shafik (1973) analyzed levels of pentaCP in human adipose tissue. In 200 mg samples of tissue, he found that most of the samples analyzed contained pentaCP ranging from 12 to 52 µg/L. Morgade et al. (1980) found a range of 10 to 80 µg/L pentaCP in 200 mg samples.

Roberts et al. (1977) conducted studies on the permeability of human epidermal membrane to chlorophenols. The permeability increased for the chlorophenols studied in the following order: 2,4,6-triCP > 2,4-diCP > 4-CP > 2-CP. The threshold concentration for 2-CP and 4-CP were 0.8 and 0.75 (%w/v) respectively. However, it was discovered that no damage was noted for 2,4-diCP or 2,4,6-triCP up to saturation. It was suggested that damage to the skin was caused by protein denaturation.

3.3. Aquatic health

The effects of chlorophenols on aquatic life have been well documented, with the majority of studies concentrating on fish toxicity. Chlorophenols have also been shown to cause tainting of fish because of bioaccumulation in the tissues.

Holcombe et al. (1980) studied 2,4-diCP toxicity to the fathead minnow. They noted an average 96 hour survival of 60% at pH 7.57 to 100% at pH 8.25 with 7.43 mg/L 2,4-diCP. At the pH range 7.57 to 7.97, most of the fish had lost equilibrium and exhibited abnormal swimming patterns after 24 hours. At 12.33 mg/L 2,4-diCP, a 12 hour survival of 0% at pH 7.47 to 100% at pH 8.81 was noted, with schooling and swimming patterns affected regardless of the pH. Because 2,4-diCP has a pKa of 7.8, less of the undissociated 2,4-diCP is present at high pH. Because it is more difficult for the ionic form to penetrate bipolar membranes the higher pH experiments showed less toxicity. Holcombe et al. (1982) then studied the effects of 2,4-diCP and pentaCP on different stages of growth of the fathead minnow (Pimephales promelas). They found a survival rate of

6.0% to 70.0% for 2,4-diCP concentrations ranging from 1240 µg/L to 290 µg/L respectively. Thus, they determined the maximum acceptable toxicant concentration (MATC) to be 290 to 460 µg/L for 2,4-diCP. PentaCP was found to be more toxic than 2,4-diCP. The survival rate ranged from 0% to 87.0% for pentaCP concentrations ranging from 223 µg/L to 73 µg/L, respectively. The MATC was determined to be 44.9 to 73.0 µg/L, an approximate five-fold increase in toxicity in comparison to 2,4-diCP. The 96 hour LC_{50} for pentaCP and 2,4-diCP were 221 µg/L and 8230 µg/L, respectively.

Buccafusco et al. (1981) investigated the toxicity of chlorophenols to bluegill (Lepomis macrochirus). The 24 hour and 96 hour LC_{50} values decreased according to the number of chlorine substituents. That is, toxicity decreased in the following order: 2,3,4,6-tetraCP > 2,3,5,6-tetraCP > 2,4,5-triCP > 2,4,6-triCP > 4-CP > 2,4-diCP > 2-CP. Hattula et al. (1981) found this same trend when trout (Salmo trutta) were exposed to chlorophenols, with pentaCP exhibiting the greatest toxicity. Konemann and Musch (1981) studied the acute lethality for guppies exposed to chloropnenols at pH values ranging from 6.1 to 7.8. Toxicity was also found to increase as the number of chlorine substitutions increased, with pentaCP the most toxic at all pH levels. Ingols et al. (1966) found that 2-CP was less toxic than 3- or 4-CP, with approximately the same toxicities when studying 24 hour tolerance levels. However, 2,4,6-triCP was 4 to 5 times more toxic than the monochlorophenols.

Some evidence has shown that there is some degree of bioconcentration of chlorophenols in fish. Rudling (1970) found from 0.2 to 3.0 mg pentaCP/kg fresh tissue in fish samples. These fish were taken from a lake that received the pentaCP-containing effluent of a pulp mill. Kobayashi et al. (1977) found that conjugated pentaCP accumulated in the bile of goldfish (Carassius auratus), and this corresponded to a concentration factor of 12,000 in 48 hours. Hattula et al. (1981) found 2,3,4,6-tetraCP and pentaCP were quite concentrated in fish tissue with values of 210 and 200 mg/kg respectively.

In addition to fish, there has been interest concerning the effects of chlorophenols on other aquatic organisms. Huang and Gloyna (1968) studied the effects of chlorophenols on algae (Chlorella pyrenoidosa). There was a positive correlation between the increase in toxicity and a higher number of chlorines on the molecule. The monochlorophenols were least destructive to chlorophyll in comparison to the polychlorinated phenols, with pentaCP being the most toxic. Also, the suppression of photosynthesis by chlorophenols was investigated. The same order of toxicity was also noted for oxygen suppression. LeBlanc (1980) studied toxicity to the water flea (Daphnia magna). Similar trends relating to increased toxicity and increased degree of chlorine substitution were not found. However, increased toxicity by chlorophenols in relation to phenol was demonstrated.

4. Physical and chemical properties contributing to persistence of chlorophenols in the environment

Table 1 summarizes the physical and chemical parameters of some selected chlorophenols. The chlorophenols are substituted phenols with from one to five chlorine substituents that impart a

Table 1. Some Physical and Chemical Properties of Selected Chlorophenols

Compound	Melting Point (°C)	Boiling Point (°C)	Density (g/L)	pKa^{f}	log K_{OW}^{f}	H (atm-m^{3}/mol)	Solubility (25°C in water)
2-CP	9.0^{b}	174.9^{e}	1.2634^{b}	8.11	2.15; 2.19		28,000 mg/L^{e}
3-CP	33^{b}	214^{b}	1.268^{b}	8.80	2.47		
4-CP	43-44^{b}	217^{d}	1.2651^{b}	9.20	2.39; 2.44	5.6 x 10^{-7c}	27,000 mg/L^{c}
2,3-diCP	57-59^{b}			7.70	3.50		
2,4-diCP	45^{b}	209-10^{d}	1.383^{e}	7.89	3.23	6.66 x 10^{-6e}	4,500 mg/L^{e}
2,5-diCP	56-58^{h}	211^{h}					
2,6-diCP	68-9^{b}	219-20^{b}		6.79			
3,4-diCP	68^{b}	253.5^{b}		8.59			
3,5-diCP	68^{b}			8.19			
2,3,4-triCP	79-81^{h}	248-9^{h}					
2,3,5-triCP	57-59^{h}						
2,3,6-triCP	55-57^{h}						
2,4,5-triCP	68-70^{b}	253^{g}	1.678^{e}	6.94	4.19	1.76 x 10^{-7e}	1200 mg/L^{e}
2,4,6-triCP	69.5^{b}	246^{b}	1.4901^{b}	5.99	3.38	9.07 x 10^{-8e}	800 mg/L^{e}
3,4,5-triCP	101^{b}	271-7^{b}		7.73	4.14		< 2 mg/L^{g}
2,3,4,6-tetraCP	69-70^{d}	164^{d}		5.40	4.42		
2,3,4,5-tetraCP				6.35	4.87		
PentaCP	188-9^{a}	310^{a}	1.978^{b}	4.74	5.01;5.24	3.4 x 10^{-6e}	80 mg/L^{g}

[a] Clayton and Clayton, 1981

[b] CRC Handbook of Chemistry and Physics, 1983

[c] Howard, 1989

[d] Lange's Handbook of Chemistry, 1973

[e] Montgomery and Welkom, 1990

[f] Woods, 1984

[g] Merck Index, 1983

[h] Aldrich Chemical Catalog, 1990

polarity to the molecule.

The acid dissociation constants (pKa) of the chlorophenols range from 4.74 to 9.20. Chlorophenols behave as weak acids, and would therefore be more soluble in basic solutions. Because the pH values found in nature vary from 6 to 9, the chlorophenols would primarily be present in equal concentrations of both the dissociated and undissociated forms, because most chlorophenols have pKa values between 6 and 9 (Krijgsheld and van der Gen, 1986). However, those with pKa values below 6 would be present in ionic form. Because 2,4-diCP has a pKa of 7.89 (Table 1), equal concentrations of both the dissociated and undissociated forms of 2,4-diCP would be found in nature, because its pKa is between 6 and 9. Because 4-CP has a pKa of 9.20 (Table 1), most of the compound would be present in undissociated form in nature.

The chlorophenols have octanol-water partition coefficients (K_{ow}) ranging from of $10^{2.15}$ to $10^{5.01}$. Mackay (1982) observed correlations between the K_{ow} and the bioaccumulation factor at K_{ow} ranges of 10^2 and 10^6, but not with compounds having K_{ow} values greater than 10^6. This correlation was observed using lipophilic organic compounds, and therefore, can be applied to chlorophenols.

4.1. Volatilization

The process of volatilization involves the transfer of a solute from water to the air-water interface. Then, the solute passes across the interface and diffuses into the air. The majority of resistance is a few millimeters above and below the interface, with the interface contributing very little or no resistance (Mackay et al., 1979).

The degree to which any compound volatilizes will depend upon several factors. These include pH, temperature, (Krijgsheld and van der Gen, 1986) water mixing, and wind velocity (Krijgsheld and van der Gen, 1986; Hutzinger and Veerkamp,1981). In addition, the presence of contaminants in natural water bodies can also affect volatilization (Cohen et al., 1978).

Mackay et al. (1979) noted that solutes with high molecular weights lead to a decrease in the liquid- (K_L) and gas-phase (K_G) mass transfer coefficients (m/h). They suggested this to be a probable result of decreased diffusivity. With solutes having a Henry's law constant (H) that was greater than 5 x 10^{-3} atm·m^3·g·mol^{-1}, the resistance was almost completely in the liquid phase. However, solutes with H less than 5 x 10^{-6} atm·m^3·g·mol^{-1} had the resistance almost completely in the gaseous phase. One problem noted for empirically determining H was that solutes had to be analyzed over a range of concentrations, and accuracy increased with higher concentrations. However, some solutes were not very soluble, and in nature, concentrations of many compounds was low. This necessitated an extrapolation which could decrease accuracy. Also, in nature, there was the possibility of interference from mineral compounds, as well as from dissolved and suspended organic material.

Cohen et al. (1978) also noted the possible interference of contaminants in the water body, but concentrated on the effects of wind velocity. They found changes in K_L depended on various wind velocities. At a velocity of less than 3 m/s, a calm surface, K_L values were strongly influenced by mixing from within the water body. At wind velocities from 3 to 10 m/s, K_L increased and was influenced by ripples and an increase in surface roughness. At wind speeds greater than 10 m/s, wave breaking could occur, and K_L would increase largely because of factors such as an increase in surface area and the presence of spray.

Chiou et al. (1980) determined the half lives of some chlorophenols under static and stirring conditions in order to observe the correlation with the calculated half lives. For 2- and 4-CP, there was good agreement when a depth of 0.38 cm was used. For 2-CP, stirring the solution decreased the evaporation half life slightly. However, no major difference was noted between 2 mg/L and 4000 mg/L solutions of 2-CP.

4.2. Photodecomposition

Several studies have focused on the ease with which chlorophenols photodecompose, as well as the products that were formed in most cases. Barbeni et al. (1986) found that complete, rapid mineralization of the compound occurred, depending on the semiconductor used to facilitate photodecomposition. The most efficient semiconductor used was TiO_2. The semiconductor was added to solution as a particulate suspension. With this semiconductor, the half lives for the photodecomposition of 4-CP, 3,4-diCP, 2,4,5-triCP, and pentaCP were 14, 45, 55, and 20 minutes, respectively. Their corresponding initial concentrations were 6, 18, 20, and 12 mg/L, respectively. As well, the experiments were carried out in a solution pH of 3. However, if no semiconductor was present, or if the components were stirred in the dark, negligible photodecomposition occurred.

Yasuhara et al. (1977) irradiated 10 mg/L aqueous solutions of 2-, 3-, and 4-CP under a nitrogen atmosphere. They found that 4-CP was the easiest to decompose, followed by 2-CP which did not completely decompose, and lastly, 3-CP barely decomposed. During the photodecomposition of 4-CP, it was determined that phenol was a product. Photodecomposition for these compounds was shown to be a first-order reaction. When greater than 1000 mg/L hydrogen peroxide was added, however, 2-CP did decompose. Omura and Matsuura (1971) also studied the products of chlorophenol photodecomposition, however, aqueous solutions containing KOH were used as the solvent under bubbling nitrogen. Upon irradiation of 4-CP, hydroquinone and 2,4'-dihydroxydiphenyl were the major products. There was also a small amount of phenol formed. With 3-CP, resorcinol was the major product, in good agreement with Boule et al. (1982). However, when 2-CP was irradiated, no identifiable product was observed.

Boule et al. (1982) studied the products of monochlorophenol photodecomposition in aerated solution. When 2-CP was tested in its molecular form, the major photo product was pyrocatechol. However, in its anionic state, cyclopentadienoic acid was the major product. At any pH tested, 3-CP always formed resorcinol as its major product. 4-CP formed hydroquinone as the main product in aerated solution.

Hwang and Hodson (1986) studied photodecomposition in estuarine water. They found that photolysis rates were higher in summer in comparison to the rates in winter. They also found that photodecomposition rates for 2,4-diCP, 2,4,5-triCP, and pentaCP were much faster than those for phenol and 4-CP.

4.3. Adsorption

Adsorption to sediments and soils is probably the most prevalent mechanism through which chlorophenols persist in the environment. This is caused by the wide availability of adsorption sites for chlorophenols, and is probably significant in sludges because of the high organic content present.

Many adsorption studies have focused on the adsorption of chlorophenols to activated carbon. Al-Bahrani and Martin (1976) investigated the adsorption of phenol and chlorophenols to activated carbon. They noted that the hydroxyl group (-OH) on phenol may have lead to the formation of hydrogen bonding with water molecules resulting in a decrease in adsorption capacity. When studying 2-CP, they found that chlorine addition to phenol increased the adsorption capacity. They suggested that this was caused by an intramolecular bond between the hydroxyl and chlorine groups preventing hydrogen bonding with water. Thus, they concluded that hydrogen bonding with water and steric hindrance were important factors to consider in adsorption.

Martin and Al-Bahrani (1977) next studied competitive adsorption on activated carbon. In batch experiments containing solutions with 2-methylpyridine, o-cresol, and 2-CP, the equilibration capacity for each solute was impaired by the presence of the other solutes. For each solute, the adsorption capacity was 50% of that when the solute was alone in solution. In addition, adsorption was also impaired. With five solutes in one solution, the loss of adsorption capacity was enhanced. Thus, competition for adsorption sites with many solutes in solution affected adsorption capacities of all solutes.

Knettig et al. (1986) also investigated competitive adsorption on activated carbon. The adsorption capacities for chlorophenols were found to increase as both molecular weight and degree of chlorination increased, although all compounds displayed similar slopes for their isotherms indicating the same degree of adsorption. As with Martin and Al-Bahrani (1977), Knettig et al. (1986) illustrated a decrease in adsorption capacity as the number of solutes increased with as much as a 60% decrease in adsorption capacity in a 3-solute system. The stronger adsorbing compounds such as 2,4-diCP and 2,4,6-triCP could be distinguished from weaker adsorbers such as 2-CP and phenol on the basis of Freundlich parameters.

Adsorption to sediment and soil has been extensively studied in attempts to approximate adsorption phenomena in nature. Isaacson and Frink (1984) looked at fine and coarse sediment and found that there was a limited adsorption capacity for phenol, 2-CP, and 2,4-diCP, which depended upon the organic carbon content of the sediment. They also found that fine treated sediment (without labile organic matter) sorbed less phenols than the fine untreated fraction. Treatment with hydrogen peroxide was used to remove the labile organic matter. The coarse fraction did not exhibit this phenomenon. Desorption was observed to be slower than adsorption, and sorbate was irreversibly held by untreated fractions. In addition, experimental partition coefficients were much higher than those calculated based on the organic content. It was suggested that sorbate-sorbent interactions may be more important than general hydrophobic interactions, because the predicted partition coefficients which were based on hydrophobic forces were much lower than those actually observed.

Boyd (1982) concentrated on the effects of functional groups on adsorption in an anaerobic system. It was noted that a solute's affinity for soil could affect its bioactivity, persistence, mobility, and volatility. If a chlorine atom was present in the o-, m-, or p-position, an increase in adsorption was seen in comparison to phenol as Knettig et al. (1986) also noted with activated carbon as the sorbent. Also, the o- isomer adsorbed less to soil than did the p-isomer, and it was suggested that this was caused by steric hindrance by the ortho chlorine of hydrogen bonding involving the phenolic hydroxyl group. Thus, it was concluded that the m- and p-isomers behaved as proton acceptors in order to hydrogen bond to soil sites. Di- and trichlorophenols had K_{OC} (soil/sediment partition coefficient) values that were greater than predicted, possibly as a result of hydrogen bonding.

Woods (1984) studied adsorption of chlorophenols at pH 7.1 to anaerobic reactor solids. Because of a disparity between the adsorption of pentaCP and 2,3,4,5-tetraCP based on the K_{OW} values, it was concluded that the pH of the solvent was an important factor to consider in adsorption. Partition coefficients were also determined from pH 6.3 to 7.8, the pH range found in anaerobic treatment. The effect of pH was pronounced at the pKa of the chlorophenol. The determined partition coefficients for chlorophenols were greater for the molecular forms than the ionized forms. Also, an increase in pH resulted in a decrease in adsorption of chlorophenols by sludge solids. The ratio of the partition coefficients for the molecular and ionic forms was found to increase with an increase in degree of chlorination.

Chlorophenols have been observed to bind to sediment in natural waterways. Wegman and van den Broek (1983) found 2,5-diCP, 2,3,5-triCP, 2,4,5-triCP, 2,3,4,5-tetraCP, 2,3,4,6-tetraCP, and pentaCP 100% of the time in sediment. Eder and Weber (1980) noted that pentaCP was the predominant chlorophenol and was always found in conjunction with 2,3,4,6- and/or 2,3,5,6-tetraCP. Comparing the levels of chlorophenols in particulates and water, the particulates had higher levels than the water. Adsorption ranged from approximately 40% for 2,4,6-triCP to approximately 70% for 2,4-diCP. However, the amounts and types of chlorophenols found in natural waters would depend on the types of discharges entering the waterways.

5. Biodegradation of chlorophenols

In the subsequent sections, the terms biotransformation and biodegradation will be used. Biotransformation will refer to the formation of another compound from the parent compound. Biodegradation will refer to the complete mineralization of the parent compound to gaseous products.

Biodegradation of chlorophenols has been shown to be difficult because of the addition of chlorine substituents to phenol (Reineke, 1984). Although, aerobic biodegradation proceeds with greater ease than anaerobic biodegradation. Basically, during biotransformation of chlorophenols, products are formed that may not be as toxic as the parent compound, as with partial biodegradation, or the compound is completely mineralized to carbon dioxide and water. However, in the former case, there can be the undesirable effect of producing an altered product that is more toxic than the original compound (Schraa and Zehnder, 1986).

Several factors may affect whether or not chlorophenols will be biodegraded. As well, the rate of biodegradation may also be affected. These factors are as follows: (Pitter, 1976; Hutzinger and Veerkamp, 1981).

1) Chemical factors such as solubility, size of the molecule, the type, number, and position of substituents, and stereochemistry which is largely determined by the substituents.
2) Biological factors such as age, manner, and time of adaptation of the microbial culture, degree of toxicity of the compound to the microbial culture, and the kinds and numbers of microbes present.
3) Environmental factors such as pH, temperature, dissolved oxygen level, and degree of dispersion of the compound.

5.1. Aerobic biodegradation of chlorophenols

The aerobic biodegradation of chlorophenols in soil, aquatic, and wastewater systems is well-documented. Generally, the monochlorophenols are more easily degraded than the more highly chlorine-substituted chlorophenols.

5.1.1. Soil

Alexander and Lustigman (1966) studied the effect of chlorine substitution in the meta, ortho, and para positions of phenol. They found that both 3- and 2-CP persisted for greater than 64 days. However, 4-CP was degraded in only 16 days. Phenol was quickly degraded in comparison to the chlorinated phenols, demonstrating the resistance of chlorophenols because of the chlorine substituent. Haller (1978) used soil as the inoculum to study the biodegradation of 2-, 3-, and 4-CP. The 4-CP was completely degraded in 14 to 25 days, but the 2- and 3-CP were not degraded after more than 25 days. These results agreed with Alexander and Lustigman (1966). Baker and Mayfield (1980) also studied aerobic degradation of chlorophenols in soil. They found that the more highly chlorinated phenols were increasingly recalcitrant in comparison to the monochlorophenols. However, 3-CP was an exception because it took 80 to 160 days to demonstrate ≥ 70% degradation, whereas 2- and 4-CP took a maximum of one and two days respectively to degrade to the same extent. In addition, a lag phase was not evident for the 2- and 4-CP, suggesting an inoculum with a pre-enriched population of chlorophenol-degraders. Scow et al. (1986) added 2,4-diCP to soil at both high (5.0 μg/g) and low (4 ng/g) concentrations. At the lower concentration, mineralization was immediate. With the higher concentration, increased lag phases were evident.

Pritchard et al. (1987) determined that the presence of sediment enhanced the degradation of 4-CP. They found that the addition of non-sterile sediment to 4-CP-containing estuarine water resulted in 25% of the 4-CP being mineralized to $^{14}CO_2$, whereas, without the sediment addition, degradation was slow or missing. Also, the biodegradation rate was proportional to the amount of sediment added. If sterile sediment, sand, or glass beads were added, a degradation rate higher than that seen in only estuarine water was also noted, suggesting the importance of a surface area in biodegradation. The presence of chlorophenol-degrading microbes was also vital for biodegradation to occur.

It was determined that factors such as redox potential and pH were also important in the biodegradation of pentaCP (DeLaune et al., 1983). DeLaune et al. (1983) studied the conditions under which pentaCP was degraded in sediment. They found that the highest degradation rates were obtained with the highest redox potentials which would be found under aerobic conditions. Also, a pH of 8 and the highest redox value of +500 mV was found to be optimum for the biodegradation rate.

5.1.2. Water

Aquatic studies concerning the aerobic biodegradation of chlorophenols have also noted a similar persistence because of the chlorine substitutions. Liu et al. (1981) studied pentaCP degradation in an aquatic environment. They found that the concentration of pentaCP decreased to a negligible amount in three days under aerobic conditions. They also noted that the addition of glucose or sodium 4-chlorophenate inhibited degradation rather than acting as inducers as was originally believed. Studies by de Kreuk and Hanstveit (1981) concentrated on differences in biodegradation of chlorophenols in fresh water and sea water. There was no trend concerning degradability that was evident. That is, 2,6-diCP was degraded more quickly in sea water, whereas, 2,4,6-triCP degraded more quickly in fresh water. Also, 2,4,5-triCP was not degraded at all in sea water. In addition, low concentrations of nutrients corresponded to low degradation rates.

Some studies have concentrated on aerobic chlorophenol degradation enhanced by the presence of additional substrates that are easier to degrade. Shimp and Pfaender (1985) found that the degradation rate of 4-CP differed when the inoculum was adapted to amino acids, fatty acids, or carbohydrates, all of which are natural substrates. The rate constant for 4-CP increased significantly as these natural substrate concentrations increased. In subsequent studies, Shimp and Pfaender (1987) also studied the effect of inoculum adapted to phenol on the degradation rate of 4-CP. As with the earlier studies, they again found significantly higher degradation rates for 4-CP. They also found a higher proportion of 4-CP degraders in the microbial population.

5.1.3. Wastewaters and sludges

Some studies have been conducted using wastewaters or sludges as the inoculum in an oxygenated system. Bunch and

Chambers (1967) used settled sewage as the inoculum in an aerobic, static system to study the degradation of phenol, 3-CP, and 2,4-diCP at concentrations of 20 mg/L. They observed rapid adaptation and degradation of phenol, but 3-CP was not degraded significantly, even after three subcultures. With 2,4-diCP as the substrate, a gradual adaptation was noted, and by the third subculture, an average of 98.5% of the compound was degraded in a week. Pitter (1976) used an open system with activated sludge as the inoculum. In this case, degradation rate was analyzed through chemical oxygen demand (COD) loss. The substrates studied were phenol, 2-CP, 4-CP, and 2,4-diCP, each at an equivalent concentration of 200 mg/L COD. The biodegradation rates increased in the following order: phenol > 2-CP > 4-CP > 2,4-diCP. In this study, an increase in chlorine substituents appeared to hinder the biodegradation rate.

Tabak et al. (1981) used settled domestic wastewater as their inoculum to study the biodegradation of phenol, 2-CP, 2,4-diCP, 2,4,6-triCP, and pentaCP. They found significant biodegradation along with rapid acclimation for all substrates except pentaCP. These substrates were 83% to 100% degraded after one week of incubation in the dark. Pentachlorophenol needed two weeks (5 mg/L) and four weeks (10 mg/L) until adaptation occurred. Again, it was noted that the more highly chlorinated phenols resulted in increased persistence. Haller (1978) used sludge supernatant as a 2.5% (by volume) inoculum to investigate the degradation of 2-, 3-, and 4-CP. All compounds were tested at a concentration of 16 mg/L. A period of 7 to 14 days was required for complete degradation of 4-CP, but both 2-, and 3-CP needed 14 to 25 days. This was in contrast to

Pitter (1976) who found that 2-CP was degraded more rapidly than 4-CP.

5.1.4. Aerobic biodegradation pathway

Chlorophenols can be aerobically biodegraded by one of two mechanisms. Either the halogen substituent is replaced with a hydroxyl group after ring cleavage, or the ring is first cleaved, then dehalogenated (Berry et al., 1987). The cleavage is accomplished through enzymes known as mono- and dioxygenases, and oxygen is assimilated into the resultant products (Evans and Fuchs, 1988).

The function of the oxygenases is to incorporate oxygen into the chlorophenol. An example of the aerobic pathway illustrating this for 2- and 4-CP can be seen in Figure 2 (Steiert and Crawford, 1985). The monooxygenase inserts a hydroxyl group adjacent to the hydroxyl group already present, producing the corresponding catechol (Steiert and Crawford, 1985; Paris et al., 1982). Ring cleavage proceeds when the dioxygenase inserts molecular oxygen into the ring. A lactonizing enzyme leads to an unstable intermediate. At this point, the chlorine is released. The lactone is cleaved by a hydrolase resulting in maleylacetate which can enter the tricarboxylic acid (TCA) cycle (Steiert and Crawford, 1985).

The presence of the chlorine substituents interferes with the function of the oxygenases by deactivating the ring, making it more difficult for the oxygen to insert into the ring. Also, as the number of chlorine substituents increases, this effect is more pronounced, which is the reason for the persistence of more highly chlorinated

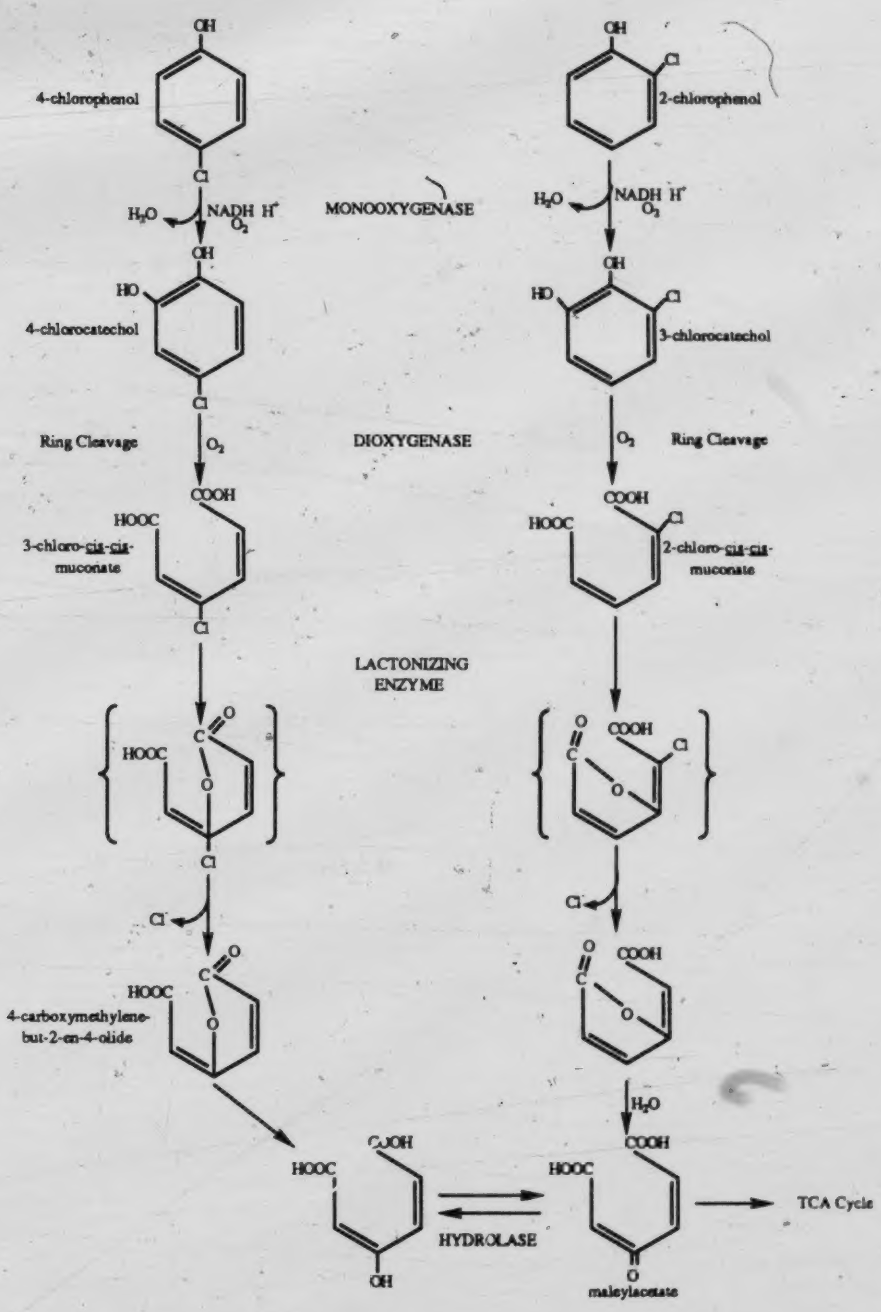
OH
4-chlorophenol
Cl
OH
Cl
2-chlorophenol
H$_2$O
NADH H$^+$
O$_2$
MONOOXYGENASE
H$_2$O
NADH H$^+$
O$_2$
OH
HO
4-chlorocatechol
Cl
OH
HO
Cl
3-chlorocatechol
Ring Cleavage
O$_2$
DIOXYGENASE
O$_2$
Ring Cleavage
COOH
HOOC
3-chloro-cis-cis-muconate
Cl
COOH
HOOC
Cl
2-chloro-cis-cis-muconate
LACTONIZING ENZYME
C
O
HOOC
O
Cl
O
C
COOH
Cl
O
Cl$^-$
Cl$^-$
C
O
HOOC
4-carboxymethylene-but-2-en-4-olide
O
O
C
COOH
O
H$_2$O
HOOC
COOH
OH
HYDROLASE
HOOC
COOH
O
maleylacetate
TCA Cycle

Figure 2. Aerobic Biodegradation Pathway of 2- and 4-Chlorophenol
(Adapted from Steiert and Crawford, 1985)

phenols, even under aerobic conditions (Steiert and Crawford, 1985; Wood, 1982).

5.2. Anaerobic biodegradation of chlorophenols

Biodegradation of chlorophenols is much more difficult under anaerobic conditions in comparison to aerobic conditions. This is primarily because of the low microbial populations present and to the unfavorable energy conditions present. That is, because the alternate electron acceptors are not as powerful oxidizing agents as oxygen, a low energy yield results (Ingraham et al., 1983). The reduction of carbon dioxide to methane results in a redox potential of -250 mV, compared to a redox potential of +810 mV for the reduction of oxygen to water (Schraa, 1988).

5.2.1. Anaerobic environments

The anaerobic environments that can be identified in nature are numerous. They include impacted and waterlogged soils, sediments, and landfills (Young, 1984; Berry et al., 1987). Anoxic aquatic sites include some groundwater, and fresh and ocean water (Berry et al., 1987). In addition, the rumen and animal intestinal tract is anaerobic (Berry et al., 1987). Sewage treatment plant digestors, and industrial plants producing methane from organic waste products are also anaerobic environments (Evans and Fuchs, 1988). The anaerobic environment, which was the focus in this thesis, was the sewage digestor.

5.2.2. Anaerobic methanogenic biodegradation processes

Anaerobic digestion involves three interrelated steps. First the complex organics are hydrolyzed and fermented by facultative anaerobes into fatty acids. Second, the fatty acids are oxidized to produce hydrogen gas and acetate, reactions known as dehydrogenation and acetogenesis, respectively. The last step is methanogenesis, the formation of methane (McCarty and Smith, 1986) Figure 3 illustrates these steps as well as the interdependent relationships among the bacteria. This figure illustrates the percentages and directions of electron flow during anaerobic digestion.

Anaerobic biodegradation is the result of a close syntrophic relationship among four groups of bacteria. The first group is the acid forming bacteria which oxidize organic matter such as carbohydrates and proteins, resulting in the formation of acids such as acetic and propionic acid. The second group is referred to as the acetogenic bacteria. This group produces acetic acid from butyric and propionic acid. The last two groups are both methanogens and are referred to as the acetoclastic and H_2-utilizing bacteria. The acetoclastic group produces methane and carbon dioxide by cleaving acetic acid. The H_2-utilizing group combines hydrogen and carbon dioxide to form methane and water (Mosey, 1982; McCarty and Smith, 1986).

An example of the anaerobic methanogenic biodegradation process can be illustrated with ethanol as the substrate. The Gibbs free energy ($\Delta G^{\circ\prime}$) for the net equation for the process is negative (Table 2). Thus, the reaction is thermodynamically favorable at a pH

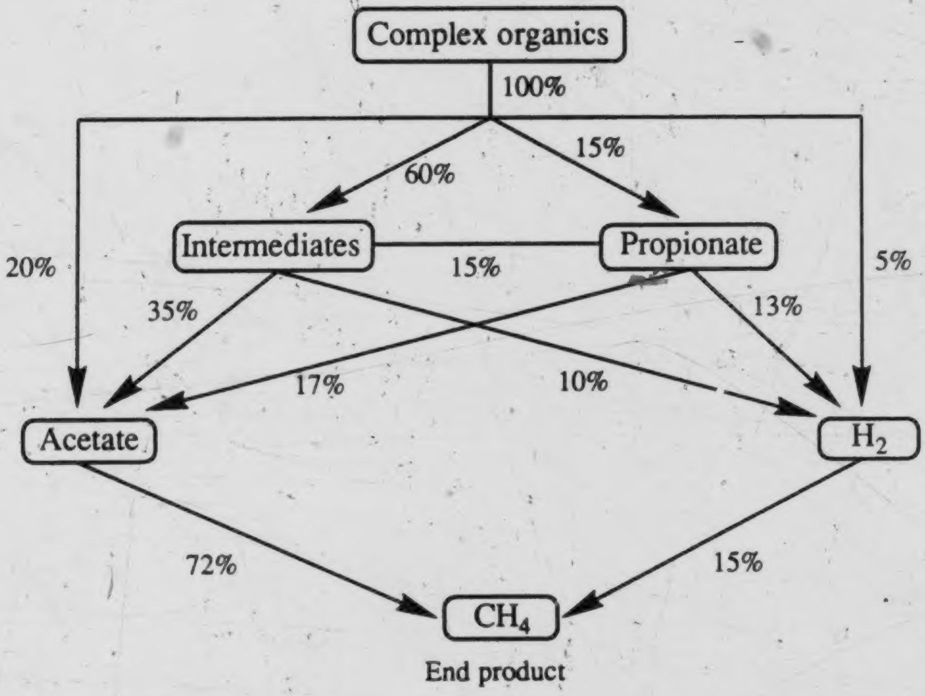
Complex organics
100%
60%
15%
Intermediates
15%
Propionate
20%
5%
35%
13%
17%
10%
Acetate
H_2
72%
15%
CH_4
End product

Figure 3. Steps Involved in Anaerobic Digestion
(Adapted from McCarty and Smith, 1986)

Table 2. Anaerobic Conversion of Ethanol to Methane*

	$\Delta G^{\circ\prime}$ (kJ)
Ethanol (Substrate)	
$CH_3CH_2OH(aq) + H_2O(l) = CH_3COO^-(aq) + H^+(aq) + 2H_2(g)$	9.65
Hydrogen	
$2H_2(g) + 0.5CO_2(g) = 0.5CH_4(g) + H_2O(l)$	-65.37
Acetate	
$CH_3COO^-(aq) + H^+(aq) = CH_4(g) + CO_2(g)$	-35.83
Net reaction for anaerobic conversion	
$CH_3CH_2OH(aq) = 1.5CH_4(g) + 0.5CH_2(g)$	-91.55

* Adapted from McCarty and Smith, 1986

of 7. However, because ΔG°' for the conversion of ethanol is positive, the concentration of hydrogen gas and acetate must be reduced by methanogens so that the free energy is negative (McCarty and Smith, 1986). An increase in acetic acid within a culture will decrease the pH of the solution, and in turn, slow down the activity of bacteria involved in methanogenesis such as Methanosarcina barkeri (an acetoclastic methane bacterium and Methanospirillum hungatei (an H_2-utilizing methane bacterium) (Mosey, 1982).

5.2.3. Biodegradation in soil and sediment

Anaerobic biodegradation of chlorophenols has been noted in soil and sediment, however, degradation is often very slow. Baker and Mayfield (1980) studied the biodegradation of chlorophenols under anaerobic conditions in batch culture at 23°C with a nitrogen atmosphere. They found no significant differences in the percent degradation between the anaerobic and sterile (non-viable) soil samples. They therefore concluded that the chlorophenols were not degraded to an appreciable degree under anaerobic conditions. In another study, they directly observed the microorganisms on soil slides containing three zones, labelled the outer-aerobic, middle-transition, and inner-anaerobic. Microbial cells in each zone were counted after the addition of phenol and some chlorophenols. The transition zone showed an increase of more than 100 cells per field when 2-CP, 2,4-diCP, and 2,6-diCP were added. However, the microbial numbers did not show a substantial increase when chlorophenols were added to the inner-anaerobic zone. Baker et al. (1980) again studied biodegradation of chlorophenols in sediment, but the sediment was at a temperature of 0°C. When they added 2-CP, 3-CP, and 2,4-diCP to the anaerobic zone, increases of 15, 30, and less than 5 cells per field were noted, respectively. However, if 4-CP was added, the microbial numbers appeared to decrease, suggesting that 4-CP was toxic to the microbes.

Smith and Novak (1987) observed biodegradation in subsurface soils. Soils were obtained from depths of approximately 4 and 30 meters. Compounds tested were phenol, 2-CP, 2,4-diCP, 2,4,6-tri CP, and pentaCP with concentrations greater than 50 mg/L, except for 2,4-diCP which was less than 50 mg/L. However, the incubation temperature throughout the experiment was 10°C. Subsequently, the compounds were significantly degraded after approximately two months, demonstrating first-order degradation kinetics. Of the compounds studied, both phenol and 2-CP displayed the highest degradation rates, followed by 2,4,6-triCP, pentaCP, and 2,4-diCP. With respect to chlorination, a higher degree of chlorination did not correlate to a higher degree of resistance.

Gibson and Suflita (1986) incubated freshwater sediment at room temperature under anaerobic conditions to study the biodegradation of chlorophenols. The atmosphere used to maintain the batch cultures was 90% nitrogen-10% hydrogen. The monochlorophenols as well as 2,4-, 2,5-, and 3,4-diCP, and 2,4,5-triCP were added at concentrations of 300 to 500 µM (48.9 mg/L to 81.5 mg/L for the diCP's and 59.24 mg/L to 98.73 for the triCP). All substrates exhibited 98% or greater removal after a period of three months incubation.

Inherent problems occur when studying biodegradation of compounds in soil and sediment. Adsorption to soil particles could provide the appearance of biodegradation. Smith and Novak (1987) noted that an initial rapid decrease in concentration occurred, attributed to both adsorption and biodegradation. Smith and Novak (1987) also found the biodegradation rates dependent on the type of soil utilized. They did find a difference in the nitrate levels present, with the higher nitrate levels enhancing biodegradation.

5.2.4. Biodegradation in sludge

5.2.4.1. Persistence

Anaerobic biodegradation of chlorophenols in sewage sludge has been extensively studied only recently. Johnson and Young (1983) tested 2-CP, 2,4-diCP, 2,4,6-triCP, and phenol in anaerobic cultures to determine the degree to which each compound inhibited anaerobic biodegradation. The inoculum used was from a digestor designed to represent municipal solid waste. Of these compounds, they found that 2,4-diCP at a concentration of 100 mg/L resulted in significant inhibition of methane production, although this was after an incubation period of only five hours. In the next set of experiments designed to further study methane inhibition, serum bottles were monitored for a maximum period of 20 days, and thus it was concluded that the substrate was inhibitory. In another experiment, removal of 2,4-diCP from cultures was followed for a period of 10 days with the conclusion that no biodegradation had occurred.

Further studies dealing with anaerobic biodegradation of chlorophenols allowed longer periods of time to pass in order to determine whether or not a particular substrate was biodegradable.

Guthrie et al. (1984) acclimated sludge to a maximum concentration of 6000 µg/L of pentaCP over a five month period. Because the methane production in reactors supplemented with pentaCP was essentially the same as the controls, they concluded that pentaCP removal was occurring. This was because Guthrie et al. (1984) considered pentaCP to be toxic to methanogens. Thus, they expected methane levels to be lower than the controls if pentaCP was not being removed.

Some researchers have concentrated on biodegradation of both the monochlorophenols and dichlorophenols. Boyd et al. (1983) studied the monochlorophenol isomers as well as phenol. They found that 2-CP and 3-CP were completely degraded in 3 and 7 weeks, respectively, in unacclimated sludge inoculum. 4-CP was found to be the most persistent of the three isomers because significant degradation did not occur during the 8 week test period. Although, an additional 8 weeks of incubation resulted in nearly complete degradation of the compound. Boyd and Shelton (1984) studied both mono- and dichlorophenol isomers in unacclimated sludge. Again, as previously shown for the monochlorophenols (Boyd et al., 1983), the rate of biodegradation was in the following order: 2-CP > 3-CP > 4-CP in a 10% sludge inoculum. The 2-CP did not exhibit a lag, whereas the 3-CP and 4-CP did not exhibit pronounced degradation until 4 weeks of incubation. They also noted that the dichlorophenols without a chlorine ortho to the hydroxyl group persisted over the 6 week incubation period.

Mikesell and Boyd (1986) also studied pentaCP degradation using sludges that were acclimated to monochlorophenols for more than two years. A concentration of 10 mg/L pentaCP was rapidly dechlorinated within three days when using the sludge acclimated to 2-CP. However, in the sludges acclimated to 3-CP and 4-CP, pentaCP was dechlorinated in 12 and 9 days, respectively. The differences in dechlorination rates may be the result of specific populations of microbes that act on a limited substrate range. In order to prove that mineralization was occurring, ^{14}C-pentaCP was added to cultures, and the presence of $^{14}CO_2$ and $^{14}CH_4$ was detected.

Similarly, Hrudey et al. (1987a) studied biodegradation of the monochlorophenols. In batch cultures, 2-CP was added up to a concentration of 285 mg/L which needed 33 weeks for complete 2-CP dechlorination, and 27 additional weeks for complete degradation of the subsequent phenol that was formed. They also determined that 3- and 4-CP at concentrations up to 100 mg/L were more persistent in comparison to 2-CP. A period of 16 to 20 weeks passed until degradation was noted. However, in the cases where complete disappearance of the substrate occurred, adding the substrate to the initial concentration resulted in more rapid degradation, indicative of adaptation to the substrate.

Hrudey et al. (1987b) added activated carbon to determine whether or not the activated carbon enhanced biodegradation by providing a more stable environment. They added activated carbon in amounts of 0, 100, 300, or 1000 mg/L to sludge cultures containing 2,3-diCP. During the initial few weeks, low levels of 2,3-diCP were observed in the supernatant. However, in the next 16 weeks, 2,3-diCP was not evident in any of the activated carbon cultures. After the 18th week, the level of 2,3-diCP was equivalent to the feed rate in all cultures except those with 1000 mg/L activated carbon. Although, this did occur in the latter cultures when the feed rate was increased. It was noted though that in all cultures, observed 3-CP production was lower than theoretical and was further reduced to almost non-detectable levels in activated carbon cultures, demonstrating removal of 3-CP.

5.2.4.2. Cross acclimation studies

Some researchers acclimated sludges to particular chlorophenols in an attempt to encourage biodegradation of related chlorophenols. Boyd and Shelton (1984) enriched three sludges to one of 2-CP, 3-CP, or 4-CP. If the sludge was acclimated to 2-CP, 2-CP and 4-CP were degraded at the same rate in the 30 day incubation period, and 16 to 18 mg/L was completely degraded in 3 to 4 days, but 3-CP was not degraded to a great extent. Of the dichlorophenols tested, only 2,4-diCP was degraded, but at a lower rate. Sludge that was acclimated to 3-CP degraded 4-CP at a lower rate than 3-CP, but did not degrade 2-CP. The diCP's 3,5-diCP and 3,4-diCP were degraded in the 3-CP enrichment cultures within 2 weeks although these same compounds persisted in unacclimated sludge for 6 weeks. The degradation rates in the 3-CP acclimated sludge were 3-CP > 3,5-diCP > 4-CP > 3,4-diCP. Thus, it was noted that chlorophenols with chlorine substituents in the meta or para positions were degraded. Sludge acclimated to 4-CP displayed the following degradation rates: 4-CP > 3-CP > 2-CP > 3,4-diCP > 2,4-diCP. Therefore, cross acclimation between subtrates with ortho and para subsituents was seen in 2-CP acclimated sludge, and cross acclimation between substrates with meta and para substituents was seen in 3-CP acclimated sludge. The 4-CP acclimated sludge was the only sludge that degraded all monochlorophenols, and it was suggested that this sludge had a wider substrate spectrum.

5.2.4.3. Inhibition

Hrudey et al. (1987a) supplemented sludge cultures containing one of the three monochlorophenol isomers with phenol to determine their effects on phenol biodegradation. They found that the addition of phenol to cultures containing 2-CP reduced the degradation rate of 2-CP. The degree to which the reduced degradation occurred was dependent on the amount of phenol added. Both 3-CP and 4-CP inhibited phenol degradation which was evident by increasing lag periods with increasing concentrations of the chlorophenols.

5.2.4.4. Adsorption to sludge

The apparent biodegradation of chlorophenols may actually be caused by adsorption of the substrates to sludge particles. Johnson and Young (1983) noted that adsorption played an important role in the removal of chlorophenol from solution. The cultures consisted of a 40% inoculum. A concentration of 100 mg/L of 2,4-diCP decreased rapidly to 60 mg/L in the first few hours of incubation. This rapid decrease was attributed to adsorption. They also determined whether or not the amount of solids present affected degradation. In cultures with 150 mg/L 2,4-diCP, the anaerobic seed plus 0, 1, or 5 g of additional solids was added. They found that the highest amount of solids did result in a decrease in toxicity, however, in none of the cultures, was there complete recovery of methane production. Thus, it was determined that the higher levels of solids reduced toxicity via adsorption.

5.2.5. Proposed stoichiometry

The following equation known as the Buswell equation was developed by Buswell and Mueller (1952) to determine the stoichiometry for complete mineralization of organic compounds:

$$C_nH_aO_b + \left\{n - \frac{a}{4} - \frac{b}{2}\right\} H_2O \longrightarrow$$

$$\left\{\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right\} CO_2 + \left\{\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right\} CH_4$$

Shelton and Tiedje (1984a) used a 10% sludge inoculum from a primary digestor to test the biodegradability of some organic compounds based on this equation. They found that 2-CP resulted in greater than 75% of theoretical methane production after 2 months incubation. Some of the non-chlorinated organic compounds that also resulted in greater than 75% of theoretical methane production using the same inoculum were 2-nitrophenol and 2-methoxyphenol. The same degree of theoretical methane production was also observed for phenol and benzoic acid using the same sludge dilution with sludge from a secondary digestor.

5.2.6. Reductive dechlorination of chlorophenols

Suflita et al. (1982) noted the importance of reductive dehalogenation to reduce the toxicity of foreign compounds that could be destructive to the environment. They found that sludge enriched on 3-chlorobenzoate (3-CB) specifically dehalogenated at the meta position.

The reductive dechlorination of chlorophenols has been well-documented. The position of the chlorine substituent is important when determining whether or not dechlorination will occur. In most cases, a chlorine ortho to the hydroxyl group was preferentially dechlorinated (Boyd and Shelton, 1984; Mikesell and Boyd, 1985; Mikesell and Boyd, 1986; Hrudey et al., 1987b). However, the earlier work by Boyd et al. (1983) did not find this preference.

Boyd et al. (1983) noted that phenol was formed as a result of dechlorination in the ortho position of 2-CP, confirmed by mass spectrometry. Hrudey et al. (1987a) also noted the dechlorination of 2-CP, forming phenol. They also found that the presence of phenol in cultures containing 2-CP inhibited dechlorination. A dechlorination rate of 0.29 mg/day in cultures containing 97 mg/L 2-CP decreased five fold to 0.057 mg/day when 86 mg/L phenol was added. It was noted that initially, the formation and accumulation of phenol in 2-CP cultures was rate limiting to dechlorination, and as the phenol concentration decreased, the dechlorination reaction in turn became rate limiting.

It has also been shown that the ortho chlorine is preferentially dechlorinated in multi-chlorinated phenols. Hrudey et al. (1987b) noted that 2,6-diCP was completely dechlorinated after one week, demonstrated by phenol accumulation, with a dechlorination rate of 0.17 mg/day. Both 2,3- and 2,4-diCP displayed rapid ortho dechlorination with 3- and 4-CP accumulating, respectively. In this case, 2,4-diCP had a dechlorination rate of 0.24 mg/day although 2,3-diCP only had a rate of 0.1 mg/day. The accumulated monochlorophenols were present in quantities that were less than predicted based on the amount of dichlorophenol initially added.

In cross acclimation studies, Boyd and Shelton (1984) found specific dechlorination patterns in sludges. Sludge acclimated to 2-CP exhibited an ortho-para dechlorination pattern, whereas sludge acclimated to 3-CP exhibited a meta-para dechlorination pattern. However, sludge acclimated to 4-CP, also dechlorinated at the ortho and meta positions. Thus, it was suggested that reductive dechlorination resulted from a specific microbiological enzymatic process or from a non-specific process conducted through microbially generated electron carriers.

Mikesell and Boyd (1986) specifically studied pentaCP dechlorination by sludges acclimated to each of the three monochlorophenol isomers. They found that each of the acclimated sludges dechlorinated pentaCP at the same chlorine position to which the sludge was initially acclimated. However, 2-CP acclimated sludge also dechlorinated at the para position, exhibiting an ortho-para pattern. Also, 4-CP acclimated sludge dechlorinated at the ortho position. The 3-CP acclimated sludge only dechlorinated at the meta position. This was in contrast to Boyd and Shelton (1984) who found a meta-para dechlorination pattern with 3-CP acclimated sludge. Mikesell and Boyd (1986) also noted that sludge simultaneously acclimated to all three monochlorophenol isomers was capable of completely dechlorinating pentaCP. In an earlier study, Mikesell and Boyd (1985) studied dechlorination of pentaCP in fresh sludge. They found that 3,4,5-triCP was rapidly formed as a result of the loss of the ortho chlorines. Also, 2,4,6-triCP formed 4-CP after losing its ortho chlorines.

5.2.7. Pathway for anaerobic biodegradation

The anaerobic biodegradation pathway for chlorophenols has not been as clearly elucidated as the aerobic pathway. As discussed in section 5.2.6., the ortho chlorine was shown to be preferentially removed. When 2-CP or 2,6-diCP was the parent substrate, phenol was positively identified as an intermediate (Boyd et al., 1983; Hrudey et al., 1987a; Hrudey et al., 1987b). Subsequently, phenol can be completely mineralized via the formation of benzoic acid or cyclohexanol as the next step in anaerobic biodegradation (Neufeld et al., 1980).

With the higher chlorinated phenols, the identification of lower chlorinated phenols caused by reductive dehalogenation has been confirmed (Boyd and Shelton, 1984; Mikesell and Boyd, 1985; Gibson and Suflita, 1986; Mikesell and Boyd, 1986; Hrudey et al., 1987a; Hrudey et al., 1987b). Gibson and Suflita (1986) noted the presence of phenol when the parent substrates were 3-CP, 4-CP, 2,4-diCP, and 2,5-diCP. However, this was in pond sediment and/or a methanogenic aquifer under anaerobic conditions. Gibson and Suflita (1986) as well as the other researchers did not positively identify phenol as an intermediate in anaerobic chlorophenol degradation except in 2-CP and 2,6-diCP degradation as previously noted. Mikesell and Boyd (1986) proposed the pathway in Figure 4, outlining the biodegradation of pentaCP. This pathway assumes that phenol was formed and degraded so rapidly that no detection was possible.

Although the identification of phenol as an intermediate has not been accomplished in sludge cultures, the complete mineralization of chlorophenols has been proven through studies with radioactively labelled substrates. Radioactively labelled pentaCP (Mikesell and Boyd, 1986), 4-CP (Boyd and Shelton, 1984; Krumme and Boyd, 1988), 2-CP, and 2,4-diCP (Boyd and Shelton, 1984) were incubated with sludge inoculum, and in all cases ^{14}C-carbon dioxide and/or ^{14}C-methane were detected in the headspace, proving that complete mineralization of chlorophenols had occurred.

6. Chlorobenzoates

6.1. Anaerobic biodegradation of chlorobenzoates

The anaerobic biodegradation of chlorobenzoates should be given some consideration because of the structural similarity of chlorobenzoates to chlorophenols. The structure of chlorobenzoates is identical to chlorophenols except that a carboxyl group (-COOH) is in place of the hydroxyl group of the chlorophenol.

Horowitz et al. (1982) found that the addition of chlorine substituents to benzoate rendered it more persistent as noted with the chlorophenols. Horowitz et al. (1982) used a 10% sludge inoculum and found that benzoate was completely mineralized in one week. Of the monochlorobenzoates studied, only 3-CB, was degraded to a maximum of 68% in 5 weeks. 3,4-Dichlorobenzoate (3,4-diCB) was

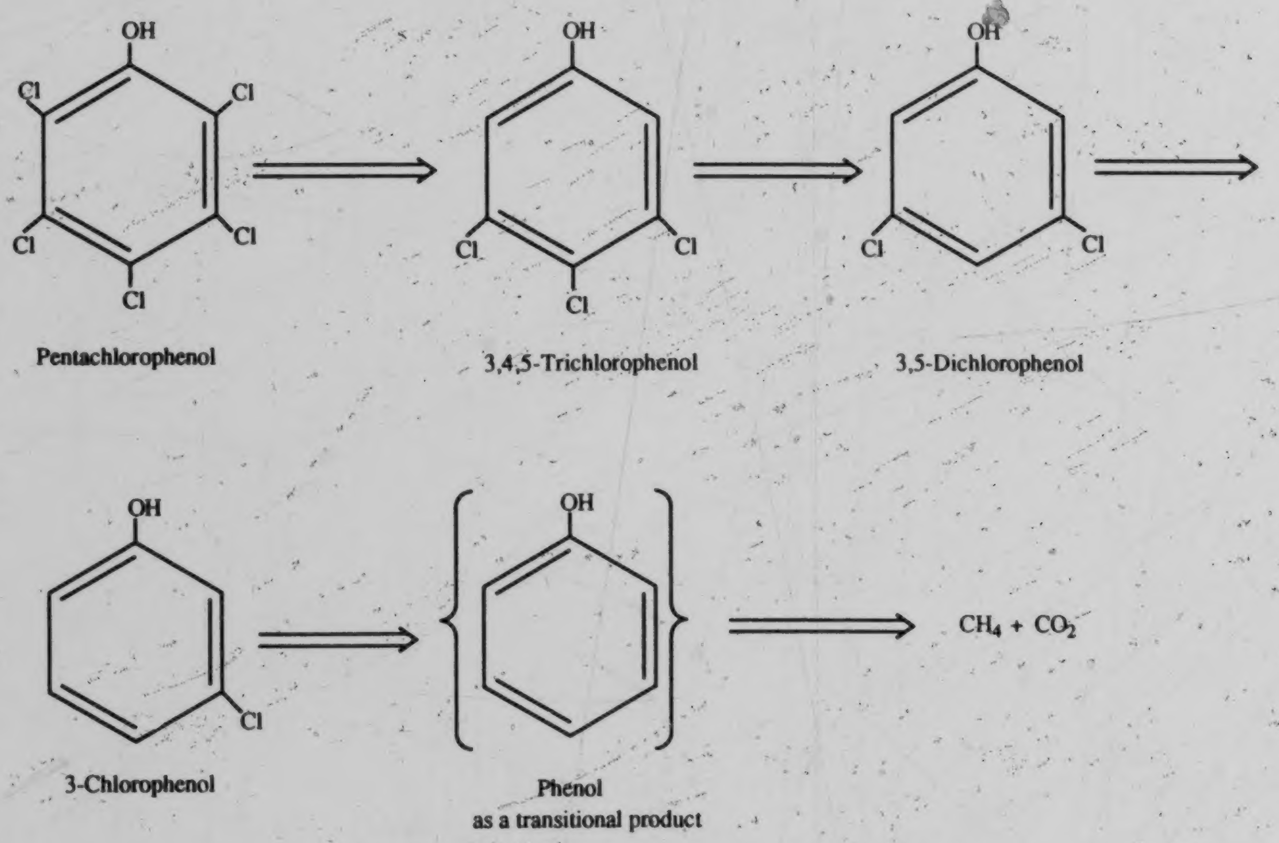
OH
Cl
Cl
Cl
Cl
Cl
Pentachlorophenol
OH
Cl
Cl
Cl
3,4,5-Trichlorophenol
OH
Cl
Cl
3,5-Dichlorophenol
OH
Cl
3-Chlorophenol
OH
Phenol
as a transitional product
CH4 + CO2

Figure 4. Anaerobic Biodegradation Pathway of Pentachlorophenol
(Adapted from Mikesell and Boyd, 1986)

not degraded after incubation for greater than 8 weeks. Suflita et al. (1982) reported the same results for the monochlorobenzoates as Horowitz et al. (1982). They also noted that 2,4-diCB and 2,6-diCB were not degraded. With the 3-CB enriched inoculum, 3,5-diCB was completely degraded. However, 2,5-diCB and 3,4-diCB resulted in 2-CB and 4-CB as products, respectively. As a result, the pathway in Figure 5 was determined for the biodegradation of 3,5-diCB.

Dolfing and Tiedje (1986) identified a food web consisting of three separate reactions carried out by three specific organisms resulting in the biodegradation of 3-CB. Shelton and Tiedje (1984b) previously identified a three member consortium, whereas Dolfing and Tiedje (1986) carried the research further. Dolfing and Tiedje (1986) found that less methane was produced during the biodegradation of 3-CB than would be expected according to the stoichiometry of the reaction. The dechlorination reaction they proposed was as follows:

$$\text{3-chlorobenzoate}^- + H_2 \longrightarrow \text{benzoate}^- + H^+ + Cl^-$$

Thus, they suggested that one third of the hydrogen produced from benzoate oxidation was recycled and used for reductive dechlorination. They proposed the following three reactions illustrating the degradation of 3-CB:

1) $\text{3-chlorobenzoate} \longrightarrow \text{benzoate} + Cl^-$
2) $\text{benzoate} \longrightarrow \text{acetate} + H_2 + CO_2$
3) $H_2 + CO_2 \longrightarrow CH_4$, and $\text{acetate} \longrightarrow CH_4 + CO_2$

The first reaction is carried out by DCB-1 (a dechlorinating organism), the second by BZ-1 (a benzoate degrading organism), and

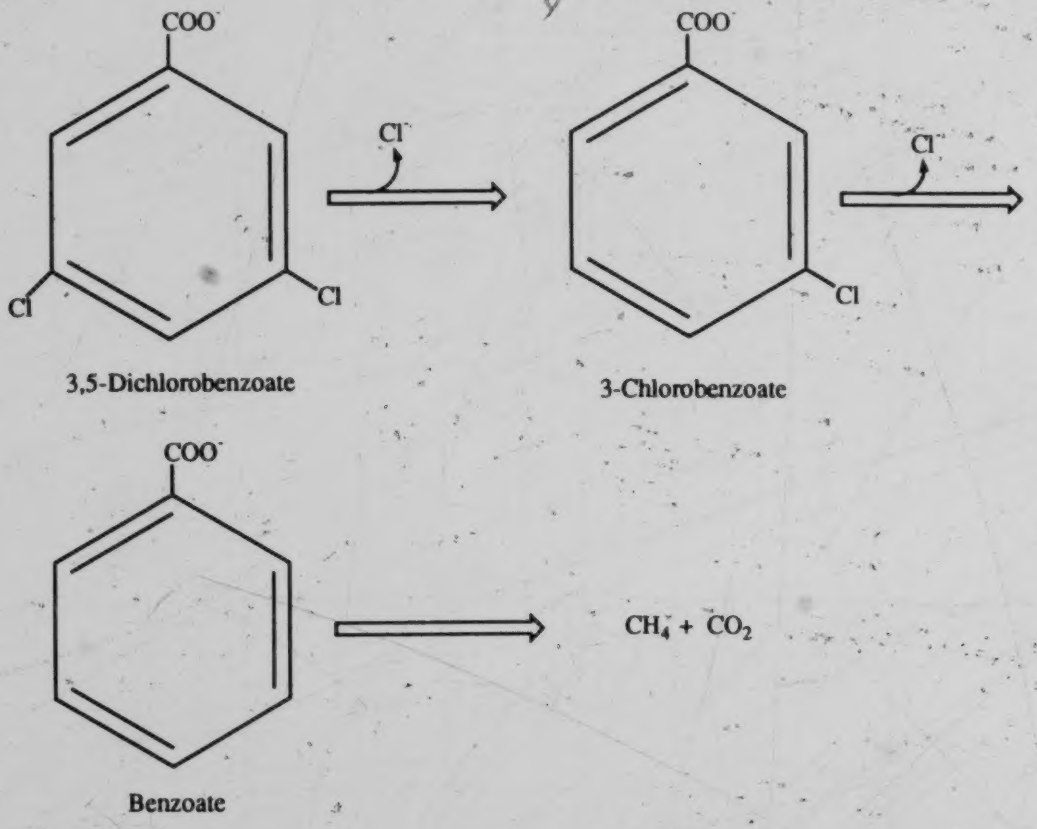
COO⁻
Cl⁻
COO⁻
Cl⁻
Cl
Cl
Cl
3,5-Dichlorobenzoate
3-Chlorobenzoate
COO⁻
CH₄ + CO₂
Benzoate

Figure 5. Anaerobic Biodegradation Pathway of 3,5-Dichlorobenzoate
(Adapted from Suflita et al., 1982)

the third by Methanospirillum strain PM-1 (a lithotrophic methanogen). The relationship among these three organisms is illustrated in Figure 6 (Dolfing and Tiedje, 1986).

6.2. Reductive dechlorination of chlorobenzoates

Reductive dechlorination has also been noted for other chlorinated substrates. These substrates primarily include the chlorobenzoates (Suflita et al., 1982; Horowitz et al., 1983; Suflita et al., 1983; Shelton and Tiedje, 1984b; van den Tweel et al., 1987; Stevens et al., 1988).

Suflita et al. (1982) enriched anaerobic sediment and sewage populations on 3-CB. They noted that reductive dechlorination occurred with many of the benzoates they studied, stating that the removal of the halogen was the initial step to complete degradation. They also found that the cultures enriched on 3-CB displayed a specificity for meta dehalogenation. Further to this work, Shelton and Tiedje (1984b) isolated a dechlorinating bacterium designated strain DCB-1. Stevens et al. (1988) noted that this was the first anaerobe isolated in pure culture that could reductively dehalogenate aromatic compounds. An assumption that Shelton and Tiedje (1984b) used to isolate DCB-1 was that benzoate or another intermediate was the source of carbon and energy because of the fact that 3-CB itself was inhibitory to the culture. They also found that pyruvate in addition to rumen fluid or a complex protein source resulted in substantial growth at 37°C. Also, without rumen fluid present, only pyruvate was able to support growth. Stevens et al. (1988) further determined that thiosulfate was stimulatory to

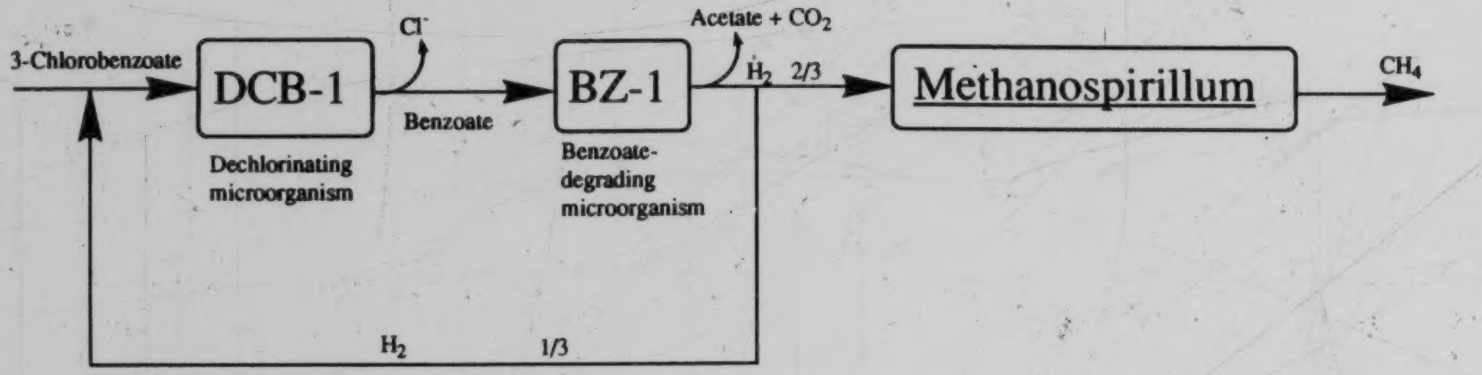
3-Chlorobenzoate
DCB-1
Dechlorinating microorganism
Cl⁻
Benzoate
BZ-1
Benzoate-degrading microorganism
Acetate + CO₂
H₂
2/3
Methanospirillum
CH₄
H₂
1/3

Figure 6. The Mutualistic Relationship Among Organisms Involved in the Degradation of 3-Chlorobenzoate (Adapted from Dolfing and Tiedje, 1986)

growth whereas sulfate was inhibitory.

Horowitz et al. (1983) observed as did Shelton and Tiedje (1984b) that the halogens were removed from chlorinated benzoates such as 3,5-diCB prior to biodegradation. They also noted that aromatic dehalogenation did not occur in the absence of methane production. Further, they found that anaerobic conditions were necessary in order for dehalogenation to proceed because it was shown that the introduction of 20% oxygen inhibited biodegradation. In addition, Marks et al. (1984) studied the dehalogenase activity of an aerobic Arthrobacter species and found that when oxygen was eliminated from the system, the observed dehalogenase activity was higher than in conditions of oxygen saturation. One unusual case was observed by van den Tweel et al. (1987). They noted that 2,4-diCB was reductively dechlorinated to 4-CB by Alcaligenes denitrificans NTB-1, an aerobic microorganism. That is, the ortho chlorine was not substituted with a hydroxyl group, the usual initial step during aerobic biodegradation of chlorinated aromatic compounds. Thus, this was the first time a catabolic route of this type was noted.

In kinetic studies with chlorobenzoates, Suflita et al. (1983) found that the Km for dehalogenation was not influenced by dilution factors. Also, they found that an additional chlorine substituent resulted in a 50% reduction in the dehalogenation rate. Suflita et al. (1982) also found a similar result in which the rate was decreased by two-thirds with the addition of a second chlorine substituent. Thus, Suflita et al. (1983) found that the Km for the dichlorinated compound was smaller than that for the monochlorinated compound. The Vmax and first-order decay constant for 4-NH_2-3,5-diCB were found to vary with dilution. That is, in diluted sediment, they were half that found in undiluted sediment samples.

7. Objectives

The objectives for this project were as follows:

1) To enrich 4-CP- and 2,4-diCP-degrading methanogenic cultures both with and without radioactive substrates for use in future experiments.

2) To determine whether or not, and to what extent, 2,4-diCP adsorbed to sterile and non-sterile sludges.

3) To prepare dilutions of sludge using both 2-CP (as a reference) and 4-CP as substrates, and determine whether dilution of inhibitory compounds that may be present would enhance degradation.

4) To determine the dechlorination rate of 2-CP as well as the amount of phenol produced during dechlorination.

5) To determine the degradation rate of 4-CP.

6) To determine, using radioactive substrates, whether 4-CP and 2,4-diCP were mineralized, and if so, determine the stoichiometry of 4-CP degradation and phenol degradation (as a reference).

7) To determine through HPLC and through fractionation of the radioactive supernatants of the 4-CP-containing cultures whether any intermediates were present.

8) To determine whether a difference in methane production (mL methane produced per mg phenol) was apparent between cultures with a low or high concentration of phenol present.

8. Methods and Materials

8.1. Analytical techniques

8.1.1. Preparation of non-radioactive standards

8.1.1.1. 4-Chlorophenol and 2,4-dichlorophenol standards

The standards for 4-CP (Fisher Scientific) and 2,4-diCP (Eastman Organic Chemicals) were prepared by weighing the appropriate amounts of the substance into vials containing a teflon liner. A 100 µL portion of 95% ethanol was added to each vial due to the low solubility of the compounds in water. The alcohol mixture was then quantitatively transferred to a volumetric flask and made up to volume with MilliQ water. The standards were placed in serum bottles which were stoppered. All standards were stored in the dark at 4°C.

8.1.1.2. Phenol and 2-chlorophenol standards

Phenol (BDH Chemicals) and 2-CP standards were stored in the same manner as the 4-CP and 2,4-diCP standards, except 95% ethanol was not added to the compound because phenol and 2-CP were soluble in water.

8.1.2. Preparation of radioactive 4-chlorophenol and 2,4-dichlorophenol standards

Uniformly ^{14}C-ring labelled phenol, 4-CP, and 2,4-diCP were received in glass ampules and prepared in 4 dram vials. The lids of the vials were fitted with teflon liners. The radioactive compound was suspended in 1 mL of distilled water and placed in a vial. The ampule was subsequently rinsed with 4 x 0.5 mL portions of water with each rinse placed in a separate vial. One of the phenol standards was rinsed with diethyl ether and evenly distributed in five vials. The ether was dried using a stream of nitrogen gas. All radioactive standards were placed in the dark at 4°C.

8.1.3. Preparation of pressure transducer standards

The pressure transducer was used to quantitate the total gas volume in the headspace. Standards used to calibrate the pressure transducer were prepared in serum bottles identical in size to the serum bottles used for the experimental cultures. The amount of distilled water added to each standard bottle was the same as the volume of liquid in the cultures. Each standard was capped with a butyl rubber serum stopper, and a needle was inserted into each serum stopper to allow the headspace of each bottle to equilibrate with the atmosphere. The needle was left in the stopper for at least 1 hour, or at the most, overnight. After equilibration, varying volumes of methane were injected into each bottle. The pressure in the headspace of the bottles was analyzed using a Microswitch 142 PC 30G pressure transducer and a 128 Digital Multimeter.

8.1.4. Analysis of 2-chlorophenol 4-chlorophenol, 2,4-dichlorophenol, and phenol in cultures

Initially, compounds were analyzed on a 2 ft x 2 mm (ID) glass column packed with 0.1% SP-1000/Carbopack C. The column was placed in a HP 5790 gas chromatograph. The oven temperature, injection temperature, and detector temperature were 210°C, 250°C, and 250°C, respectively. The nitrogen, air, and hydrogen flows were 30 mL/min, 200 mL/min, and 40 mL/min, respectively. Later, another 2 ft x 2 mm (ID) glass column was placed in the chromatograph, but was packed with 1% SP-1000/60/80 Carbopack B. After, the dilution experiment, an HP 5890 gas chromatograph was used for most of the experimental work. A 2 ft x 2 mm (ID) stainless steel column packed with 1% SP-1000/60/80 Carbopack B was placed in the HP 5890 gas chromatograph connected to a 3892A integrator. The oven temperature, injection temperature, and detector temperature were 210°C, 250°C later changed to 225°C, and 250°C respectively. The helium, air, and hydrogen flows were 60 mL/min, 427 mL/min, and 79 mL/min respectively.

Phenol concentrations in some of the phenol radioactivity recovery experiments were analyzed on a 6 ft x 1 mm (ID) stainless steel column packed with Tenax GC coated with 5% polyphenyl ether. The column was placed in an HP 5890 gas chromatograph. The oven temperature, injection temperature, and detector temperature were 200°C, 250°C, and 250°C respectively. The nitrogen, air, and hydrogen flows were 27.8 mL/min, 412 mL/min, and 37 mL/min, respectively.

The culture that was to be sampled for analysis of phenols was inverted until the solids had settled. While the bottle was inverted, a Hamilton syringe was carefully inserted into the stopper, and a 1 µL sample of the supernatant was removed. The excess liquid was wiped off the tip of the syringe, and the plunger was pulled back so that a small amount of air was introduced into the tip of the syringe. The sample was then injected into the gas chromatograph.

8.1.5. Gas proportional counter

8.1.5.1. Gas proportional counter instrumentation

The $^{14}CH_4$ and $^{14}CO_2$ were separated on a Varian Aerograph 700 GC containing a 9 ft x 5 mm column with Poropak R as the packing material. The GC contained a thermal conductivity detector that operated at 24°C and 150 mV. The propane (quench gas) flow was 12 to 13 mL/min and the helium (carrier gas) flow was 120 to 125 mL/min. The GC oven and injector temperatures were 60°C and 24°C, respectively. The GC was in turn connected to a gas proportional counter (GPC) Packard Model 894. The gas proportional counter was used to determine the number of beta particles emitted by ^{14}C. The beta particles ionized the propane gas in the proportional counter tube, and electrons as well as positively charged gas ions were generated. The electrons moved towards the positive end of the tube and a pulse of current was produced. The pulse activated a counter and was recorded. The GPC operated at 1750 mV, the current filament was set at 150 mV, the time constant was set at 20, and the HV was set at 1950 volts. Peaks were recorded on a model 7127A strip chart recorder attached to the GPC.

8.1.5.2. Preparation of standards for the gas proportional counter

A stock solution of ^{14}C carbon dioxide was prepared by placing 5 mL of 4 N sulfuric acid in a 38 mL serum bottle and capping it with a butyl rubber serum stopper. An amount of ^{14}C bicarbonate was injected into the bottle to generate the radioactive carbon dioxide. A known volume of radioactive carbon dioxide was injected into the GC attached to the gas proportional counter using a Lo Dose 0.5 mL syringe. The same volume injected was also injected into at least 4 capped 38 mL serum bottles which were later flushed to quantitatively recover and determine the amount of radioactivity in the sample added to the serum bottle.

8.1.5.3. Analysis of $^{14}CH_4$ and $^{14}CO_2$ using the gas proportional counter

8.1.5.3.1. Determination of gas proportional counter efficiency

To determine the actual DPM of methane or carbon dioxide within the headspace, a baseline was visually drawn through the background trace. The height of each peak was measured from the baseline to the tip of the peak and recorded in mm. The width at half height was determined by measuring from the inside of the right of the line to the outside of the left of the peak so that the width of the line used to draw the peak was not measured twice.

The efficiency of the gas proportional counter was determined based on the standards as follows:

$$\text{Efficiency} = \frac{\text{Range x Peak ht(mm) x Peak width at 1/2ht (mm)}}{\text{Average DPM of 4 bottles}} \times \frac{\text{100/Ht of chart paper(mm) x Chart spd(mm/min)}}{\text{20 mL/Total flow(mL/min)}}$$

The efficiency of the gas proportional counter should be greater than or equal to 70%.

8.1.5.3.2. Determination of $^{14}CH_4$ and $^{14}CO_2$ in the headspace from recorded peaks

A volume of 0.1 mL of the headspace of the experimental culture was injected into the GC attached to the GPC using a Lo Dose 0.5 mL syringe. A trace of the headspace was recorded on chart paper. To determine the amount of radioactive methane or carbon dioxide in the headspace of a particular culture, three injections of the headspace were analyzed.

To determine the actual DPM of radioactive methane or carbon dioxide in the headspace, each peak was recorded in the same manner as the standards. The efficiency was used to determine the DPM as follows:

$$\text{DPM} = \frac{\text{Range x Peak ht(mm) x Peak width at half ht(mm)}}{\text{Average efficiency}}$$

$$\text{x } \frac{\text{100/Ht of chart paper(mm) x Chart spd(mm/min)}}{\text{20 mL/Total flow(mL/min)}}.$$

8.1.6. Determination of non-radioactive methane in the headspace

A volume of 0.1 mL of the headspace of the culture to be analyzed was removed after the culture was at room temperature. The headspace was removed using a Lo Dose 0.5 mL syringe that was rinsed three times with carbon dioxide. The 0.1 mL volume was injected into a Microtek MT-220 gas chromatograph containing a 6 ft x 2 mm glass column that was packed with GP 10% SP-1000/1% H_3PO_4 on 100/120 chromosorb WAW (Supelco). The GC was attached to a 3390A integrator. The nitrogen, air, and hydrogen flows were 20 mL/min, 300 mL/min, and 30 mL/min. The percentage of methane present was determined based upon a calibration curve. The volume of methane present was determined by using both the calibration curve and pressure transducer analyses. Also, the room temperature, barometric pressure, and water vapor pressure were taken into account to calculate the actual volume of methane in the headspace.

The carbon dioxide used to rinse the syringe was obtained by placing some dry ice into a 500 mL Erlenmeyer flask. The flask was sealed with a rubber stopper with two pieces of tubing. One piece was open at one end and used to vent pressure, and the other piece was capped and used to remove the carbon dioxide.

8.2. Experimental techniques

8.2.1. Characteristics of sludge inoculum

8.2.1.1. Source

All sludge was obtained from the anaerobic sludge digestor at the Goldbar Wastewater Treatment Plant in Edmonton, Alberta. The sludge obtained was kept at room temperature overnight with the lid loosened to let gases escape. Because the sludge was used at the latest the following day, it was referred to as fresh sludge.

8.2.1.2. Total suspended solids

One piece of glass fiber filter (Whatman, GFC/1) was placed in an aluminum dish (3 for each sample) and heated in a 550°C furnace for half an hour. Each dish was taken out and kept at room temperature for 15 minutes and then placed in a dessicator for at least half an hour or overnight. The dish was then weighed to the nearest 0.0001 g.

Using tweezers, a filter was placed in a vacuum filtration apparatus, the filter was wetted with a small amount of distilled water, and suction was applied. A 2 to 5 mL sample of sludge was dispensed onto the filter using a wide mouth pipet and then filtered. The filter container and filter were washed with three 3 to 5 mL portions of distilled water. After the excess water on the filter was removed, the filter was removed using tweezers and placed back in the aluminum dish. The dish was dried in a 103°C to 105°C oven for 1 hour. The dish was then placed in a dessicator to cool for at least half an hour. When cool, the dish was reweighed to the nearest 0.0001 g.

The total suspended solids (TSS) were calculated as follows:

$$\text{mg total suspended solids/L} = \frac{(A - B) \times 1000}{\text{sample volume, mL}}$$

where,

A = weight of aluminum dish + filter + residue, mg

B = weight of aluminum dish + filter, mg.

8.2.1.3. Volatile suspended solids

The aluminum dish containing the filter and residue from the total suspended solids analysis was ignited at 550°C for 1 hour. The dish was kept at room temperature for 15 minutes and then placed in a dessicator for at least half an hour. The dish was then weighed to the nearest 0.0001 g.

The volatile suspended solids (VSS) were calculated as follows:

$$\text{mg volatile suspended solids/L} = \frac{(A - B) \times 1000}{\text{sample volume, mL}}$$

where,

A = weight of aluminum dish + filter + ignited residue, mg

B = weight of aluminum dish + filter + residue, mg.

8.2.1.4. Total solids

An evaporating dish was dried at 550°C ± 50°C in a muffle furnace for 1 hour. The dish was then placed in a dessicator until needed. The dish was weighed to the nearest 0.0001 g immediately before use.

A 5 mL sample was placed in the evaporating dish. The dish with the sample was placed on a steam bath, and the sample was evaporated to dryness. The evaporated sample was dried for at least 1 hour in an oven at 103°C to 105°C. The dish was cooled in a dessicator and weighed to the nearest 0.0001 g.

The total solids (TS) were calculated as follows:

$$\text{mg total solids/L} = \frac{(A - B) \times 1000}{\text{sample volume, mL}}$$

where,

A = weight of dried residue + evaporating dish, mg

B = weight of evaporating dish, mg.

This procedure as well as those described in section 8.2.1.2. and 8.2.1.3. were based on similar procedures in Standard Methods (1985).

8.2.2. Preparation of 4-chlorophenol-degrading and 2,4-dichlorophenol-degrading enrichment cultures

In order to prepare enrichment cultures, fresh sludge was obtained and used within 3 hours. A number of 158 mL serum bottles were gassed with O_2-free 30% carbon dioxide in nitrogen flowing through a metal canula for at least 1 minute. At the same time, the sludge was poured slowly (to minimize the amount of air that might be introduced into the sludge) into a 500 mL Erlenmeyer flask. A magnetic stirrer was added, and the sludge was allowed to stir while it was being dispensed. Also, the headspace of the flask containing the sludge was gassed while stirring by positioning another metal canula just above the mouth of the Erlenmeyer flask.

Using a 25 mL wide mouth pipet, 49 mL of sludge were dispensed into the gassing serum bottles. A cut butyl rubber serum stopper was placed over the opening of the bottle and left gassing for at least 1 minute. The canula was removed and the stopper was quickly inserted. An aluminum seal was then crimped over the stopper.

The substrates were prepared as stock solutions so that 1 mL added to the sludge would give a final concentration of 25 mg/L. These were prepared the day before obtaining the sludge. The appropriate amount of substrate was weighed into a 15 or 20 mL screw cap test tube. While gassing, the correct amount of freshly boiled, deoxygenated water was added, and the solution was autoclaved for 20 minutes.

The substrate was added using a 1 mL syringe. The syringe was gassed, and 1 mL was added to the sludge cultures. The substrate was added in this manner to ensure that there would be positive pressure in the culture bottles rather than a vacuum. The cultures were incubated at 37°C without shaking.

8.2.3. Preparation of experimental cultures

8.2.3.1. Growth medium

The stock solutions used in the growth medium are shown in Table 3. The components used in the growth medium are shown in Table 4.

After the medium was prepared, it was brought to a boil, and boiled over a Bunsen burner or hot plate for at least 2 minutes to drive off the dissolved oxygen. Just after the heat was removed, 30% carbon dioxide in nitrogen was bubbled into the medium using a sparger. As the medium was cooling, 0.57 g/100 mL sodium bicarbonate was slowly added to the medium. The medium was ready for use when the pH, as determined using a pH meter, was 7.0 ± 0.1.

Serum bottles to be used for the cultures were gassed with 30% carbon dioxide in nitrogen through a metal canula for 1 minute. Appropriate volumes of the medium were added to the serum bottles as they were being gassed, and cut butyl rubber serum stoppers were placed over the bottles for 1 minute. The canula was then removed, and the stopper quickly inserted into the bottle. To keep the stopper in place, as well as tighten the seal, an aluminum seal was crimped over the stopper. The bottles containing the medium were autoclaved for 20 minutes for sterility.

Prior to inoculation, 2.5% sodium sulfide was added (final concentration of 4 mM) using a syringe rinsed in 30% carbon dioxide in nitrogen in order to reduce the medium. The sodium sulfide was prepared by dissolving the solid in the appropriate amount of freshly boiled, deoxygenated water in a screw cap test tube or in a serum bottle. Freshly boiled, deoxygenated water was prepared by boiling

Table 3. Stock Solutions Used in Growth Medium*

Stock solution	Components	Concentration in distilled water (g/L)
Mineral solution 1†	NaCl	50
	$CaCl_2.2H_2O$	10
	NH_4Cl	50
	$MgCl_2 \cdot 6H_2O$	10
Mineral solution 2	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10
	$ZnSO_4 \cdot 7H_2O$	0.1
	H_3BO_3	0.3
	$FeCl_2 \cdot 4H_2O$	1.5
	$CoCl_2 \cdot 6H_2O$	10
	$MnCl_2 \cdot 4H_2O$	0.03
	$NiCl_2 \cdot 6H_2O$	0.03
	$AlK(SO_4)_2 \cdot 12H_2O$	0.1
Vitamin B	Nicotinic acid	0.1
	Cyanocobalamine	0.1
	Thiamine	0.05
	p-Aminobenzoic acid	0.05
	Pyridoxine	0.25
	Pantothenic acid	0.025
Phosphate	KH_2PO_4	50
Resazurin		0.1

† In 0.01 M HCl instead of water

* Adapted from Fedorak and Hrudey, 1984

Table 4. Growth medium composition*

Solution	Amount
Distilled water	100 mL
Mineral solution 1	1.0 mL
Mineral solution 2	0.1 mL
Vitamin B	0.1 mL
Phosphate	1.0 mL
Resazurin	1.0 mL
Sodium bicarbonate	0.57 g

* Adapted from Fedorak and Hrudey, 1984

distilled water for at least two minutes, and placing it under an atmosphere of 30% carbon dioxide in nitrogen. The water at room temperature was added to the solid (also gassed with 30% carbon dioxide in nitrogen) and the test tube or bottle was capped.

8.2.3.2. Substrate preparation for cultures

All substrates were prepared in freshly boiled, deoxygenated water. The preparation of the freshly boiled, deoxygenated water was explained in section 8.2.3.1. The appropriate amount of substrate was weighed into either a small serum bottle or a screw top test tube. The container was gassed briefly with 30% carbon dioxide, and the proper amount of freshly boiled, deoxygenated water was added using a pipet rinsed with 30% carbon dioxide in nitrogen. The container was then capped and autoclaved for 20 minutes. Substrates were added to cultures with plastic syringes rinsed with 30% carbon dioxide in nitrogen.

8.2.3.3. Addition of inoculum to cultures

Inoculum was always added using a glass syringe. The plunger was greased in order to ensure a gas-tight seal, and the syringe was rinsed at least three times with 30% carbon dioxide in nitrogen. The inoculum was added through the stopper. The cultures were incubated at 37°C without shaking.

8.2.4. Total recovery of added radioactivity

8.2.4.1. Gas: Radioactive methane and carbon dioxide

The culture to be analyzed was removed from the incubator and placed at room temperature for at least 1 hour (for a 60 mL serum bottle). After the culture was at room temperature the pressure transducer was calibrated, and then the headspace pressures in the cultures were determined. The barometric pressure and room temperature were also determined. Three samples of the headspace gas were analyzed using the GPC to determine the amount of ^{14}C-carbon dioxide and ^{14}C-methane in the headspace.

The total DPM of each gas in the headspace was calculated as follows:

The DPM of the injected headspace volume was determined as explained in section 8.1.7. The DPM/mL in the headspace for each gas was then calculated. The actual headspace volume of the culture was calculated using the transducer readings, room temperature, and barometric pressure. The following was used to calculate the total DPM of each gas present in the headspace:

Total DPM= DPM/mL x Headspace volume (mL).

To determine the total carbon dioxide in the culture, a flushing procedure was employed. One mL of 6.2 M hydrochloric acid was added to the culture. A series of two scintillation vials containing 1 mL of Carbosorb and 10 mL of ACS fluor each were connected through plastic tubing. One end contained a needle which was inserted into the culture bottle after insuring that there was no leaking and the other end had extra tubing acting as a vent for the system. A metal canula was connected to nitrogen gas and adjusted to a flow of 100 mL/min to 120 mL/min. The metal canula was then placed in the culture bottle into the acidified solution which was then flushed into the two scintillation vials. The two vials were counted using a liquid scintillation counter (LSC) to determine the total ^{14}C-carbon dioxide in the culture.

The total carbon dioxide was calculated as follows:

Total $^{14}CO_2$ = (DPM in vial 1 + DPM in vial 2) - 2(Background DPM) + DPM removed during headspace analysis.

This procedure was based upon a similar procedure by Fedorak, et al. (1982).

8.2.4.2. Radioactivity in the supernatant

Some of the experimental cultures only had the supernatant counted, but not the washes. A wash refers to the rinsing of the 0.45 µm pore diameter filter with a 1 mL portion of distilled water. The rinse water was rinsed into a scintillation vial and 9 mL of ACS fluor was added. These vials were counted in an LSC. These were the cultures used to identify possible intermediates in 4-CP degradation. The liquid was removed by centrifuging 2 to 3 mL of the acidified culture (removed with a plastic 1 mL syringe) in a clinical centrifuge for 10 minutes. The liquid was then put through a 0.45 µm pore diameter disposable Millipore filter. Samples of the clear liquid were placed in 10 mL of ACS fluor and counted using an LSC.

The cultures used in total radioactivity recovery experiments had the washes and supernatant counted. One mL of the acidified culture was filtered, using a 1 mL plastic syringe, through a Swinex filter containing a 0.45 µm pore diameter disposable Millipore filter, and the filtrate was collected in a 20 mL scintillation vial. The filter was washed 3 to 5 times with 1 mL portions of distilled water, and each wash was collected in a scintillation vial. Ten mL of ACS fluor was added to each vial, and they were counted in a liquid scintillation counter.

The radioactivity in the liquid was determined as follows:

First, the total DPM in the washes of the 1 mL of culture that was filtered was determined as follows:

DPM/mL = (Wash 1 - Background DPM) + (Wash 2 - Background DPM) + (Wash 3 - Background DPM) + (Wash 4 - Background DPM) + (Wash 5 - Background DPM).

Next, the total DPM in the liquid was calculated as follows:

^{14}C-liquid = DPM/mL x dilution factor.

If a dilution of the culture is made prior to filtering, then a dilution factor was taken into account in the above equation.

8.2.4.3. Radioactivity in the solids

The cultures used to identify intermediates had the solids prepared using a vacuum filtration apparatus. A piece of Whatman #1 filter paper was placed in the vacuum filtration apparatus. A 0.5 mL portion of the acidified culture was filtered, and the filter was washed with three 5 mL portions of distilled water. The vacuum was operated for three minutes after the excess water from the last wash was removed, in order to dry the filter. The filter was removed using tweezers and placed in a 20 mL scintillation vial. Ten mL of ACS fluor was added to the vial and counted.

The filter in the Swinex filtration apparatus was removed using tweezers and placed in a 20 mL scintillation vial. Ten mL of fluor was added and then counted.

The radioactivity present in the solids was calculated as follows:

^{14}C-solids = (DPM on filter - Background DPM) x dilution factor.

If a dilution of the culture was made prior to filtering, then a dilution factor was taken into account in the above equation.

8.2.5. High performance liquid chromatography

8.2.5.1. High performance liquid chromatography instrumentation

A Waters HPLC system with a Waters M-45 pump was connected to a Waters Lambda-Max Model 480 LC spectrophotometer set at 210 nm. The column used to separate compounds was an HP Lichrospher 100 RP-18, 5 µm 3.9 mm x 15 cm spherical stainless steel reverse phase HPLC column. A Gilson model 201 fraction collector was used to collect fractions. The chromatograms were charted on an HP 3392A integrator attached to the detector.

8.2.5.2. Solvents used for high performance liquid chromatography

Three different solvents were used throughout this project. They are listed as follows:

I) 48% acetonitrile
4% glacial acetic acid
made up in MilliQ water

II) 24% acetonitrile
4% glacial acetic acid
made up in MilliQ water

III) 15% methanol in 75 mM ammonium phosphate buffer adjusted to pH 3 with 20% H_3PO_4.

All solvents were prepared in a 1 liter graduated cylinder. Solvents I and II were prepared by first adding the acetic acid, followed by the acetonitrile. The solution was then made up to volume with MilliQ water. Solvent III was prepared by adding the ammonium phosphate, followed by the methanol. The MilliQ water was added nearly to the 1 liter mark. The solution was then placed on a stirrer to dissolve the ammonium phosphate. After the ammonium phosphate was dissolved, 20% H_3PO_4 was added, while the solution was stirring, until the pH was 3 as determined by a pH meter. Then the solution was made up to volume with MilliQ water.

The flow rate used for all solvents was 1.0 mL/min.

8.2.6. Fractionation of samples in conjunction with high performance liquid chromatography

A portion of the sample to be fractionated was injected into the HPLC, and fractions were collected in order to determine where the majority of the radioactivity was distributed. Triplicate samples of the same volume were placed in empty scintillation vials to determine the initial radioactivity that was injected. Ten mL of ACS fluor was added, and the vials were counted in an LSC.

The fractions were collected in 13 x 100 mm test tubes in which 1.5 mL Eppendorf tubes (with the lids removed) were held. At the end of the analysis, each Eppendorf tube was removed and placed upside down in a scintillation vial containing 9 mL of ACS fluor. All fractions were then counted in a scintillation counter, and compared to a background containing 10 mL of ACS fluor.

9. Results

9.1. Gas proportional counter efficiency

The GPC efficiency was based on ^{14}C-carbon dioxide. It ranged from 69.4% to 100% with an average efficiency of 91.0% ± 7.9%.

9.2. Calibration curves for 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, and phenol

The calibration curves for 2-CP, 4-CP, and 2,4-diCP were linear up to 40 mg/L. Phenol was linear up to approximately 300 mg/L, but as discussed in section 9.18., the lower concentrations of phenol fell on a separate calibration curve than that of the higher phenol concentrations.

9.3. Enrichments of 4-chlorophenol-degrading and 2,4-dichlorophenol-degrading cultures without radioactive substrates

Several enrichment cultures with 4-CP and 2,4-diCP were prepared using sludge as the inoculum. A summary of these are shown in Table 5. The August enrichment cultures contained the highest amount of organics and biomass in relation to the other cultures. Twenty 50 mL cultures were established with each containing 48 mL sludge, 1 mL resazurin, and 1 mL substrate. All enrichment cultures were prepared to a final concentration of 25 mg/L substrate in 158 mL serum bottles. After approximately 6 months, the August enrichment cultures completely degraded the substrate. These enrichment cultures degraded the 4-CP much more quickly than the enrichment cultures prepared after August.

The November enrichment cultures contained 98 mL of sludge

Table 5. Summary of Sludge Enrichment Cultures

Sample	Average TSS (mg/L)	Average VSS (mg/L)	Substrate	Acclimation Time
August 26, 1987	3.6×10^4	1.9×10^4	4-CP	~ 6 mos.
October 9, 1987	9.3×10^3	6.2×10^3	4-CP	> 1 yr.
November 9, 1987	1.4×10^4	9.2×10^3	2,4-diCP	> 1 yr.

and 2 mL of substrate. After nearly one year, the cultures still contained detectable amounts of 4-CP, and therefore, had not depleted the first amount of substrate that they received. The October enrichment cultures were prepared as the November enrichment cultures, except that their initial substrate was 4-CP. These also contained detectable 4-CP after nearly a year of incubation.

9.4. Enrichments of 4-chlorophenol-degrading and 2,4-dichlorophenol-degrading cultures with radioactive substrates

The radioactive enrichment cultures were prepared with the sludge used for the August enrichment cultures. In addition to the amounts of sludge, resazurin and substrate as described in section 8.2.3., uniformly ^{14}C-ring labelled 4-CP or 2,4-diCP was added to the cultures so that the total DPM in each enrichment was approximately 1 x 10^6 or 2 x 10^4 DPM/mL. Subsequently, an additional equivalent amount of radioactivity was added to the enrichment cultures to a final concentration of 4 x 10^4 DPM/mL. The radioactive substrates were added in µL amounts so that the pH of the culture would not be adversely affected. Radioactivity was initially added to the cultures in an attempt to search for intermediates during 4-CP degradation.

The distribution of radioactivity throughout each culture was determined at time 0 by removing a 0.5 mL sample (immediately after adding the radioactive substrate), placing it in a test tube and centrifuging for 10 minutes in a clinical centrifuge at a speed equivalent to a g force of 1640. The results are shown in Tables 6a

Table 6a. Distribution of Radioactivity in ^{14}C-4-Chlorophenol-Containing Cultures*

Culture	Volume (mL)	Total Radioactivity	Supernatant Radioactivity	% Radioactivity in Supernatant	Solids Radioactivity	% Radioactivity in Solids
A	49.5	8.08×10^5	4.70×10^5	58.2	3.38×10^5	41.8
B	49.5	8.01×10^5	3.92×10^5	48.9	4.09×10^5	51.1
C	49.5	8.51×10^5	3.89×10^5	45.7	4.62×10^5	54.3
D	49.5	5.95×10^5	3.02×10^5	50.8	2.93×10^5	49.2
E	49.5	7.75×10^5	3.55×10^5	45.8	4.20×10^5	54.2

Table 6b. Distribution of Radioactivity in ^{14}C-2,4-Dichlorophenol-Containing Cultures*

Culture	Volume (mL)	Total Radioactivity	Supernatant Radioactivity	% Radioactivity in Supernatant	Solids Radioactivity	% Radioactivity in Solids
A	49.5	1.99×10^6	5.66×10^5	28.4	1.42×10^6	71.6
B	49.5	2.21×10^6	5.79×10^5	26.2	1.63×10^6	73.8
C	49.5	1.94×10^6	5.38×10^5	27.7	1.40×10^6	72.3
D	49.5	1.44×10^6	3.40×10^5	23.6	1.10×10^6	76.4
E	49.5	1.82×10^6	4.85×10^5	26.6	1.34×10^6	73.4

* All measurements of radioactivity in Table 6a and 6b are in DPM and were measured at time 0 (immediately after adding the radioactive substrates)

and 6b. As noted, nearly 50% of the total radioactivity added resided in the solids portion in the 4-CP-containing cultures. However, more than 70% of the total radioactivity added to the 2,4-diCP-containing cultures was found in the solids portion. The large amount of radioactivity found in the solids prompted a study of 2,4-diCP adsorption, the results of which are in section 9.5.

9.5. Adsorption of 2,4-dichlorophenol to sludge

9.5.1. Sludge adsorption I

The purpose of this experiment was to determine whether 2,4-diCP adsorbed to sludge, and, if so, to what extent. Bottles containing sludge (not sterilized) and sterile sludge were tested. The variability of replicate samples of the supernatant varied from 1 to 5 mg/L.

9.5.1.1. Sampling from and preparation of sludge (not sterilized) and sterile sludge

Three 158 mL serum bottles were prepared and contained the following: 23.8 mL growth medium, 25 mL non-sterile sludge, 0.2 mL $Na_2S \cdot 2H_2O$. 1 mL of 2,4-diCP (to a final concentration of 25 mg/L) was added after the first three components were added and the serum bottle was capped. Another two 158 mL serum bottles contained the following: 23.8 mL growth medium, 25 mL sludge (not sterilized), and 0.2 mL $Na_2S \cdot 9H_2O$. The three components in the second set of bottles were added simultaneously, and were autoclaved for 30 minutes for 2 consecutive days to sterilize them. Between each autoclaving, they were stored at 4°C. A sterile distilled water control containing the same 2,4-diCP concentration was prepared and contained the following: 49 mL water and 1 mL substrate. The water was autoclaved for 20 minutes the night before so that it had sufficient time to cool. This bottle was to represent the variation in analysis of 2,4-diCP for both the non-sterile and sterile sets of cultures.

Each of the three sterile cultures was sampled at 0 min (time of substrate addition), 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, 6 h, and 7 h. Each of the three bottles was started approximately 20 min after the previous one to allow sufficient time for the first 4 sampling times. The sampling times for the sterile water were 0 min, 30 min, 1 h, 2 h, 3 h and 43 min, 5 h and 43 min, and 7 h and 43 min.

The sampling times for the non-sterile cultures were 0 min (substrate addition), 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, and 7 h. The sampling times for the sterile water used with this set of cultures were 0 min, 30 min, 1 h, 2 h, 2 h and 20 min, 5 h and 20 min, and 7 h and 20 min.

For each sampling, 0.1 mL of solution was removed after shaking, placed in a 1.0 mL plastic centrifuge tube and centrifuged for 10 min in a Beckman Microfuge II at a speed equivalent to a g force of 11,600. The supernatant was removed after centrifuging, placed in another plastic centrifuge tube and frozen. This procedure was also carried out on the sterile water control. The samples for both the non-sterile and sterile sludges were analyzed on HPLC. Figure 7 shows the results of this experiment.

In addition, the TS and TSS of the sludge inoculum as well as

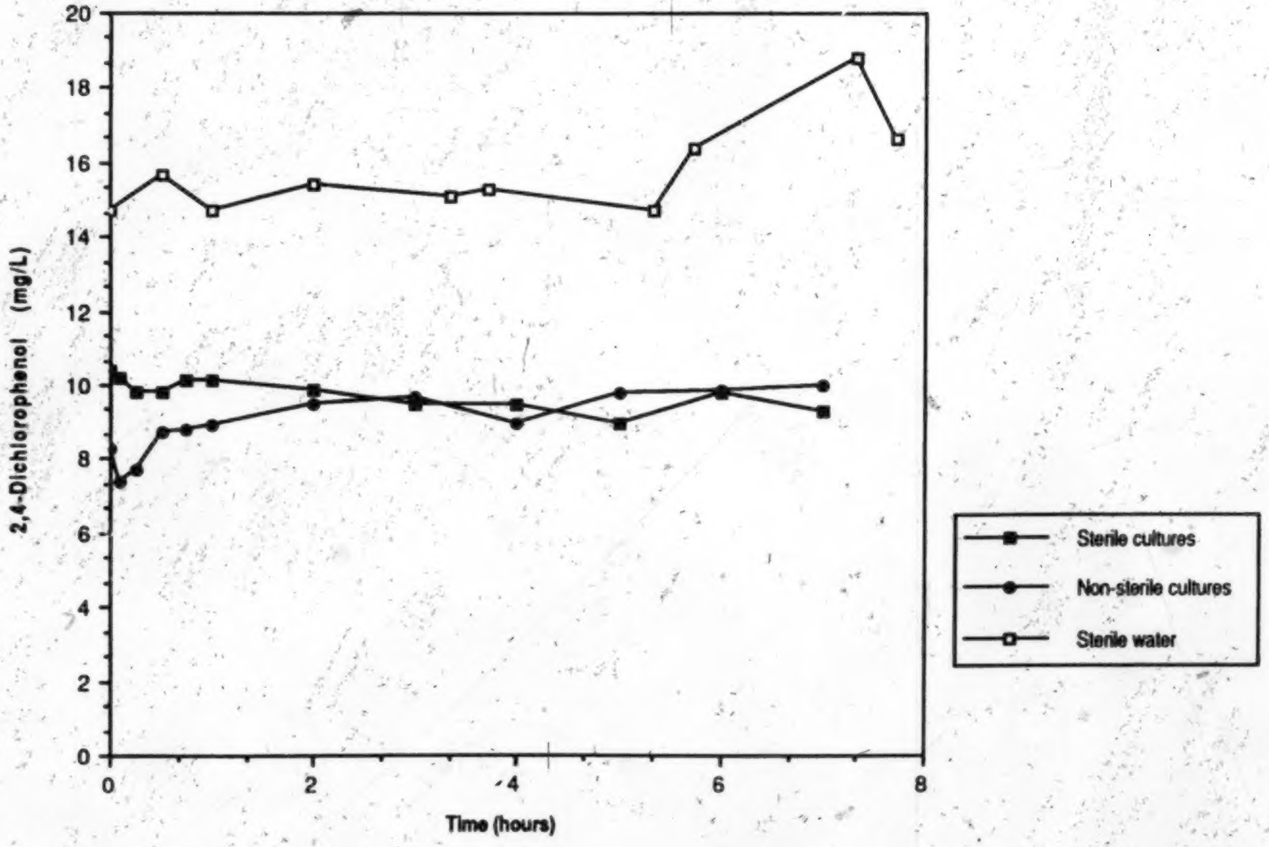

Figure 7. Adsorption of 2,4-Dichlorophenol to Sterile and Non-sterile Sludge I

the sample bottles were determined. Table 7 shows the results of these analyses.

9.5.1.2. Analysis of the 2,4-dichlorophenol stock solution used in the adsorption experiments

The concentration range for the sterile water control was approximately 16 to 18 mg/L, whereas a concentration close to 25 mg/L was expected in the experiments described in sections 9.5.1.1. and 9.5.2..

Therefore, an analysis of the 2,4-diCP stock solution added to the cultures in the adsorption experiment was carried out as follows:

a) A 1 mL sample of the stock solution was added to 49 mL of water. The concentration was analyzed on the HP 5890 gas chromatograph 10 times. After cleaning with water, the syringe was inserted and the volume to be analyzed was removed without rinsing the syringe in the solution to be analyzed. The average concentration was 22 mg/L ± 3 mg/L.

b) The same was done as in a). However, the syringe was rinsed several times with the 2,4-diCP solution, and then the volume to be analyzed was removed and analyzed. This was done 8 times, and the average concentration was 23 mg/L ± 1 mg/L.

9.5.2. Sludge adsorption II

The purpose of this experiment was also to study 2,4-diCP

Table 7. Analysis of Inoculum and Cultures From Adsorption I

Sample	Total solids in mg/L (Average ± 1 SD)	Total suspended solids in mg/L (Average ± 1 SD)
Inoculum	$1.65 \times 10^4 \pm 70$	$1.59 \times 10^4 \pm 140$
Sterile cultures	$1.05 \times 10^4 \pm 100$	$6.6 \times 10^3 \pm 100$
Non-sterile cultures	$1.07 \times 10^4 \pm 200$	$7.6 \times 10^3 \pm 200$

adsorption to sludge as was done in sludge adsorption I as described in section 9.5.1.1. However, there were some variations in the conduct of this experiment. First, the samples to be analyzed were removed directly from the bottles and analyzed via the HP 5890 gas chromatograph rather than centrifuging and then freezing the samples as done previously. Also, the bottles were shaken at 50 rpm on a desktop shaker at room temperature throughout the experiment.

The bottles with both the sterile and non-sterile sludge contained the same amounts of components as sludge adsorption I and were prepared in the same manner. However, when inoculating the non-sterile cultures, the sterile ones were also inoculated and autoclaved as described in section 9.5.1. for the sterile samples. The analysis times for all cultures were 0 min (substrate addition), 1 h, 2 h, 3 h, 4 h, and 5 h. Two additional samples were taken the following day, allowing the bottles to shake overnight. The results are plotted in Figure 8.

In addition, the TSS and VSS were determined on all bottles. The results of these analyses are in Table 8.

9.6. Dechlorination rate of 2-chlorophenol by an enriched culture

An established 2-CP digestor on a continuous feeding schedule was used as inoculum in several experiments. However, the bacterial population appeared to be inactive because 2-CP removal was no longer observed. The digestor had an average TSS of 4.2 x 10^3 mg/L and an average VSS of 2.3 x 10^3 mg/L. A month prior to the inoculation of the experiments, the activity of the digestor was determined to establish whether or not it would be suitable for use

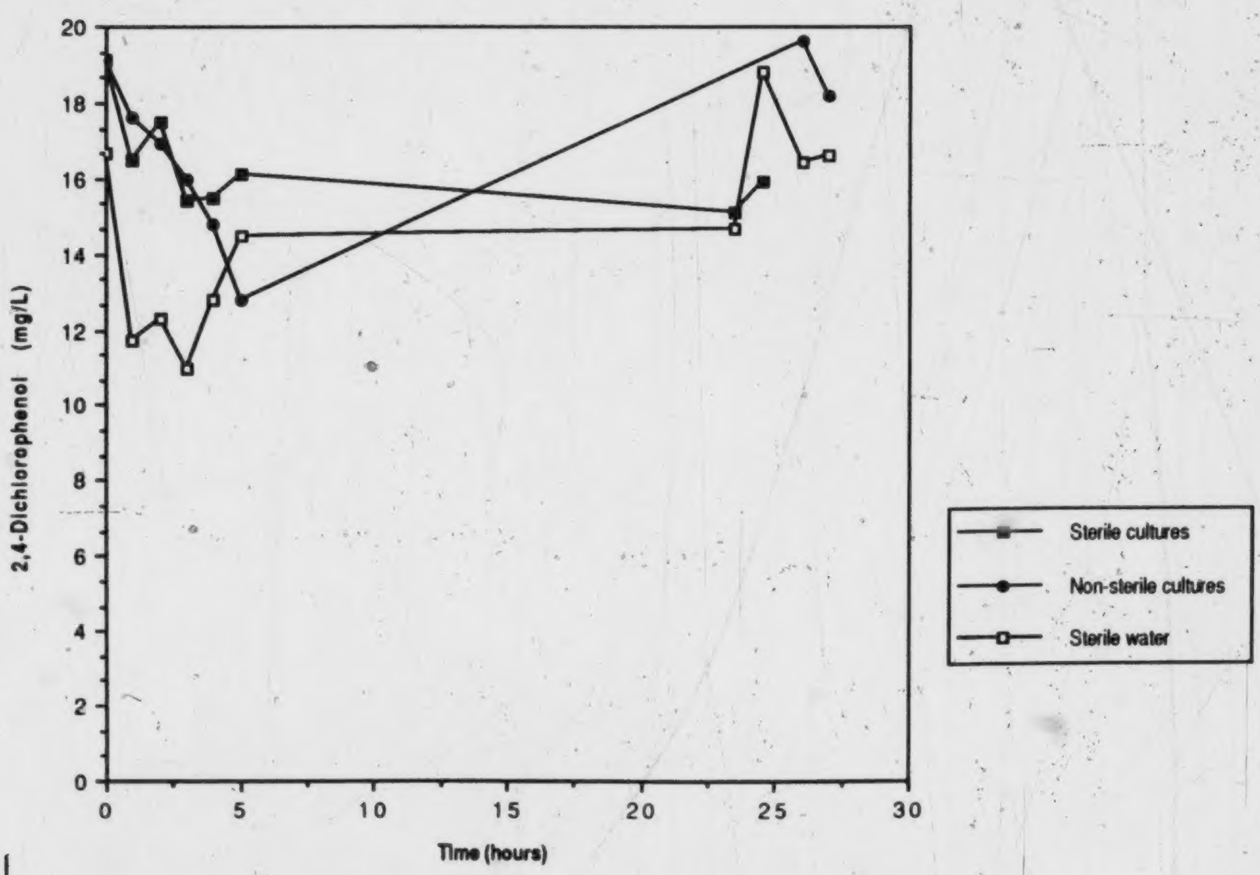

Figure 8. Adsorption of 2,4-Dichlorophenol to Sterile and Non-sterile Sludge II

Table 8. Analysis of Inoculum and Cultures From Adsorption II

Sample	Total suspended solids in mg/L (Average ± 1 SD)	Volatile suspended solids in mg/L (Average ± 1 SD)
Inoculum	$1.58 \times 10^4 \pm 30$	$9.8 \times 10^3 \pm 76$
Sterile cultures	$7.1 \times 10^3 \pm 95$	$4.03 \times 10^3 \pm 51$
Non-sterile cultures	$8 \times 10^3 \pm 140$	$4.99 \times 10^3 \pm 86$

as an inoculum. Two 158 mL serum bottles containing 25 mL digestor inoculum, 24 mL freshly boiled, deoxygenated water, and 1 mL 2-CP substrate were monitored daily. A degradation rate of 2.9 mg/L/day as determined via linear regression was observed, with the plot shown in Figure 9. This rate was referred to as the dechlorination rate since it has been proven as shown in section 9.7.2.2.1. that phenol was produced as 2-CP was dechlorinated.

9.7. Dilution of inhibitory compounds or metabolites

Initial experiments that were carried out were to observe whether a dilution of fresh sludge using freshly boiled, deoxygenated water would decrease the lag time for either 2-CP- or 4-CP-degrading cultures. This presumed that there were components within fresh sludge that may inhibit degradation. These experiments were inspired by similar research conducted by Roberts et al. (1987) on the anaerobic degradation of m-cresol.

All compounds were initially analyzed on the HP 5790 GC packed with the 0.1% SP-1000/Carbopack C column. However, due to the long retention times, a stainless steel column packed with 1% SP-1000/Carbopack B was placed in the GC midway through the experiment. Prior to measuring substrate concentrations on the new column, calibration curves for all compounds were constructed to insure linearity.

The sludge obtained had an average TSS of 5.3 x 10^3 mg/L and an average VSS of 1.9 x 10^3 mg/L. The cultures labelled normal test contained 48 mL sludge, 1 mL resazurin, and 1 mL of 2- or 4-CP substrate to a final concentration of 25 mg/L. The rest of the tests

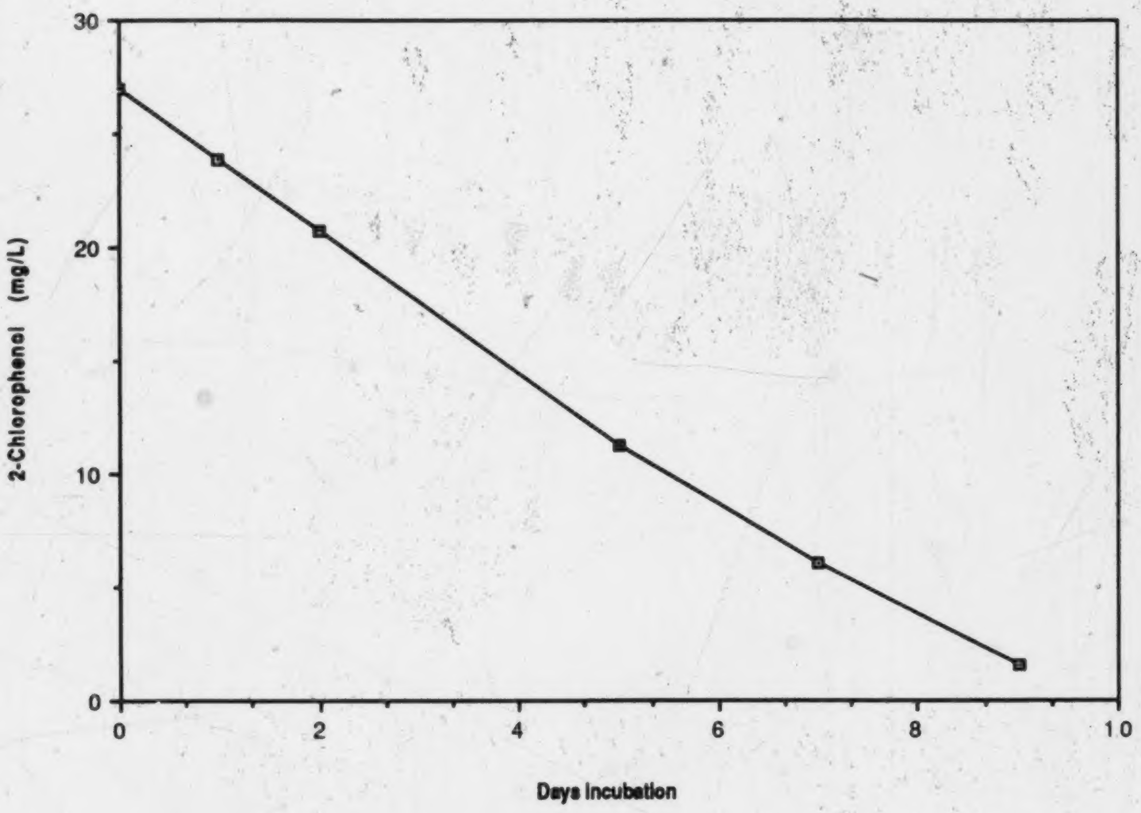

Figure 9. Dechlorination of 2-Chlorophenol by an Established Digestor

labelled 1/8, 1/4, and 1/2 contained 6, 12, and 24 mL of inoculum, respectively. They all contained the same amounts of resazurin and substrate as the normal test with enough freshly boiled, deoxygenated water added to a final volume of 50 mL. The controls for each dilution contained the same inoculum volume as well as resazurin. An extra milliliter of water was added to account for the substrate solution volume. All cultures were prepared in triplicate. Methane analysis was carried out twice a week, while the substrate was analyzed once or twice a week.

9.7.1. 4-Chlorophenol degradation and subsequent methane production

The 4-CP-containing cultures were incubated for 34 days, and when substrates were analyzed, the concentrations in all dilutions were still approximately 25 mg/L. To demonstrate whether the concentrations were the same, a Duncans multiple range test was done. Through this test, it was determined that the culture substrate concentrations were the same, and the experiment was terminated (Appendix G). The data are not shown because there was no apparent degradation.

Monitoring of the net methane production by the cultures, showed the methane did not change. As expected from the results of the Duncans multiple range test (Appendix G), there was not any significant increase in methane production noted above the controls, and the results for methane are not shown.

9.7.2. Dechlorination of 2-chlorophenol

9.7.2.1. Dechlorination of 2-chlorophenol by different sludge inocula

To determine the dechlorination rate of 2-CP, different sludge inocula were used. The inocula used were the same sludge from the 2-CP digestor as described in section 9.6., sludge from two separate phenol digestors, designated Phenol 3 and Phenol 4, and fresh sludge. The fresh sludge used had an average TSS of 7.3 x 10^3 mg/L and an average VSS of 4.8 x 10^3 mg/L.

Cultures were prepared in 58 mL serum bottles, and contained the following: 4.75 mL growth medium, 5.0 mL inoculum, 0.2 mL substrate to a final concentration of 25 mg/L, and 0.05 mL $Na_2S \cdot 9H_2O$. The cultures were only analyzed for substrate concentration.

As can be seen in Figure 10, the fresh sludge completely dechlorinated the 2-CP by day 5, demonstrating the most active microbial population. The dechlorination rate for the fresh sludge was 12 mg/L/day determined through linear regression for the 3 linear points on the plot. The other inocula were not uniformly dechlorinating 2-CP, thus their rates were not determined. Since the 2-CP acclimated digestor was exhibiting the slowest dechlorination rate, the experiments using this inoculum were terminated.

9.7.2.2. Dechlorination of 2-chlorophenol by diluted cultures

As seen in Figure 11, the 2-CP-containing cultures had increased lag periods corresponding to increased dilutions of the cultures. This was particularly apparent in the cultures diluted 1/8.

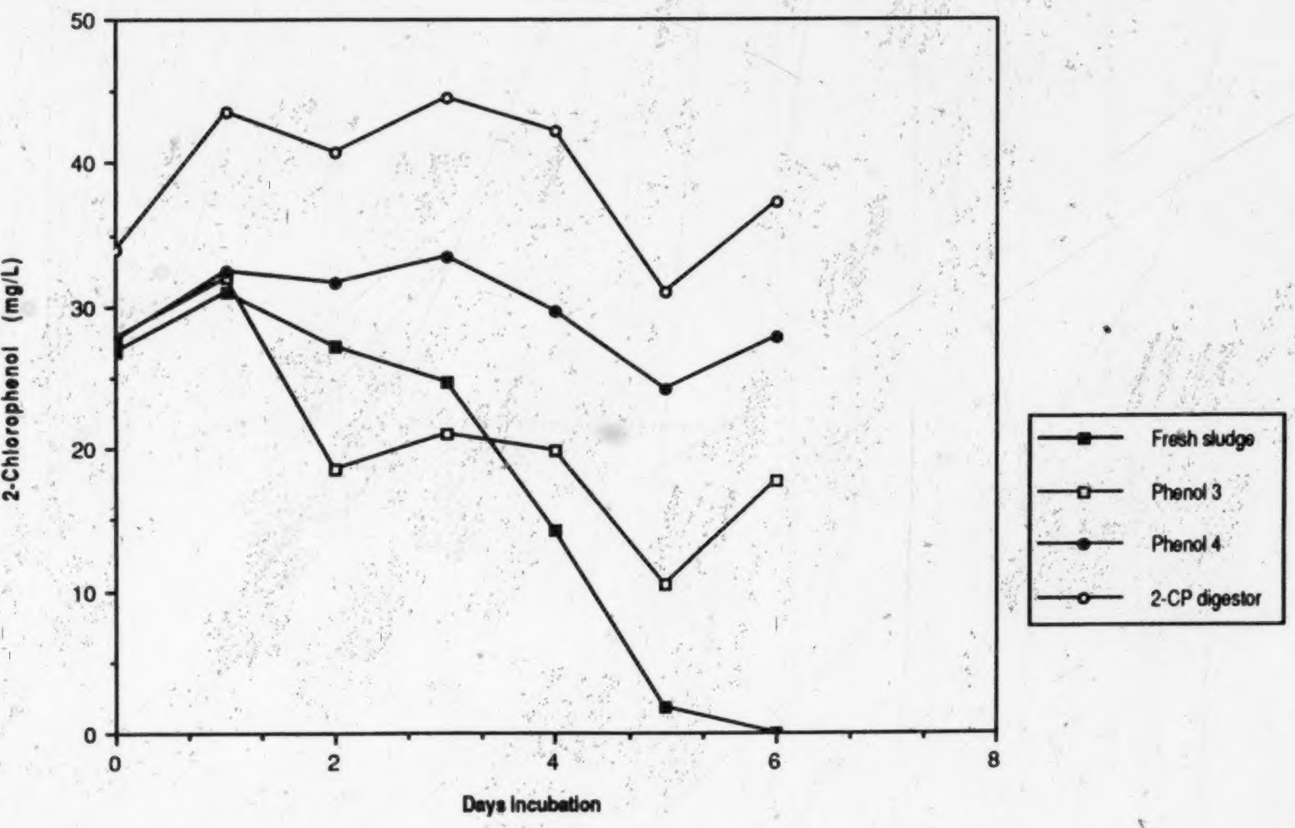

Figure 10. Dechlorination of 2-Chlorophenol Using Different Inocula

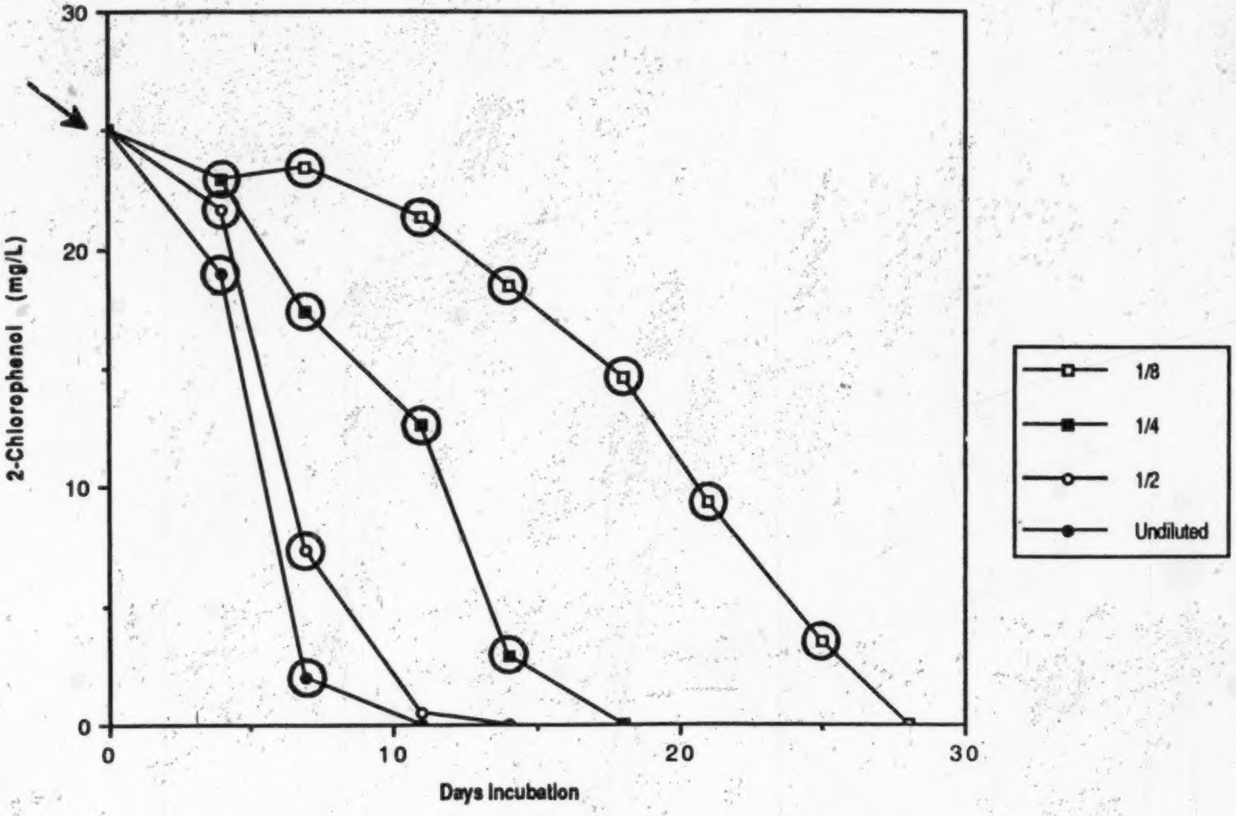

Figure 11. Dechlorination of 2-Chlorophenol by Diluted Sludge Cultures

The initial concentration of each culture was not analyzed at time 0, although the stock solution of 2-CP was prepared such that the final concentration in the cultures was 25 mg/L. The arrow at time 0 indicates that this point was not analyzed.

The average dechlorination rate for each dilution was calculated by linear regression using the linear points in Figure 11. The average rates for the 1/8, 1/4, 1/2, and undiluted culture were 1.3, 1.9, 4.8, and 5.7 mg/L/day, respectively. The number of points used to calculate these rates was 6, 4, 2, and 2 for the 1/8, 1/4, 1/2, and undiluted culture, respectively. The points used to calculate the rates are circled in Figure 11. Thus, the first rates for the 1/8 and 1/4 cultures were more accurate. However, it was evident from Figure 11 that the increased dilutions of fresh sludge resulted in decreased dechlorination rates, as well as increased lag periods.

9.7.2.2.1. Phenol production from 2-chlorophenol dechlorination

The production of phenol was observed in all of the diluted cultures (1/8, 1/4, 1/2) and the undiluted culture. The more dilute cultures produced phenol later than the other cultures, corresponding to their decreased dechlorination rate. In all cases, the phenol production began early in the incubation, within a week. Also, in all cases, phenol production was at a peak when the 2-CP concentration was below 4 mg/L. The average phenol concentration at the peak was 13.5 mg/L with a range of 12.3 mg/L to 14.0 mg/L (Figure 12). A maximum of 18.3 mg/L phenol should be produced (Appendix B). This compares with the average of 13.5 mg/L actually observed in the experimental cultures.

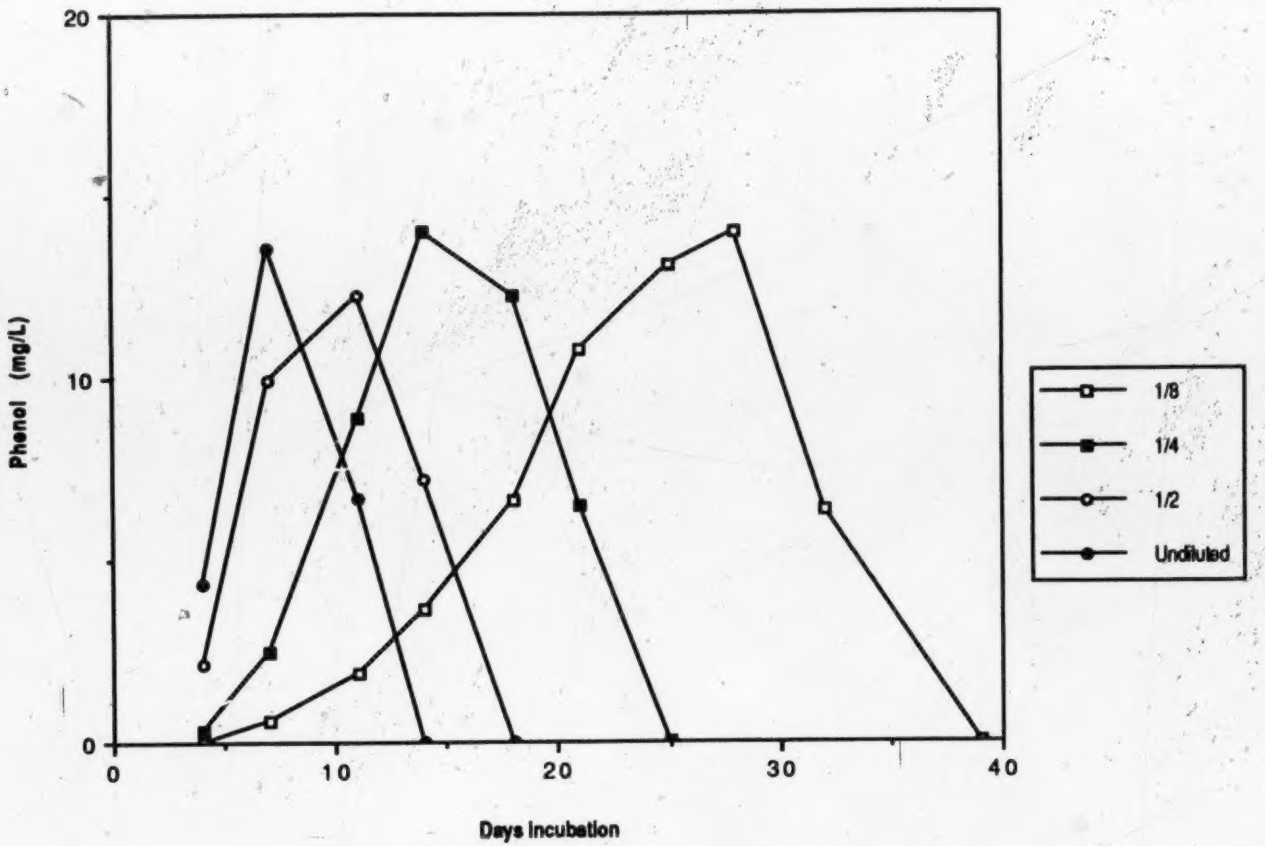

Figure 12. Phenol Production From Diluted Sludge Cultures

9.7.2.2.2. Methane production from phenol biodegradation

Very little methane production was noted for these cultures as can be seen in Figure 13. An initial increase in methane production was seen due to the degradable organics present in the sludge. By approximately day 20 of the incubation, there was a slight increase in the amount of methane produced which then eventually levelled. In most cases, the methane production even appeared to drop slightly. Theoretically, 0.7 mL of methane should be produced based on 0.75 mL methane produced/mg phenol (Appendix C) if an initial concentration of 25 mg/L 2-CP was added to the cultures initially.

9.8. Enrichment cultures containing radioactive substrates

9.8.1. Analysis of ^{14}C-4-chlorophenol- and ^{14}C-2,4-dichlorophenol-containing cultures for $^{14}CO_2$ and $^{14}CH_4$

All enrichment cultures containing radioactive 4-CP and 2,4-diCP were inoculated in August, 1987 and analyzed for radioactive gas production after 6 months incubation. At this time, there was a problem with the efficiency of the GPC which averaged 36%. However, although quantitation of the gases was possible, the accuracy of any results calculated using such a low efficiency would be highly questionable. Thus, samples of the headspace gases of all 10 cultures (5 with ^{14}C-4-CP and 5 with ^{14}C-2,4-diCP) were analyzed. The volumes used were 0.5 to 1.0 mL. Triplicate analyses were done for each culture. In all 10 cases, ^{14}C-methane and ^{14}C-carbon dioxide were qualitatively identified. This was the first instance in which mineralization products of 4-CP and 2,4-diCP were observed.

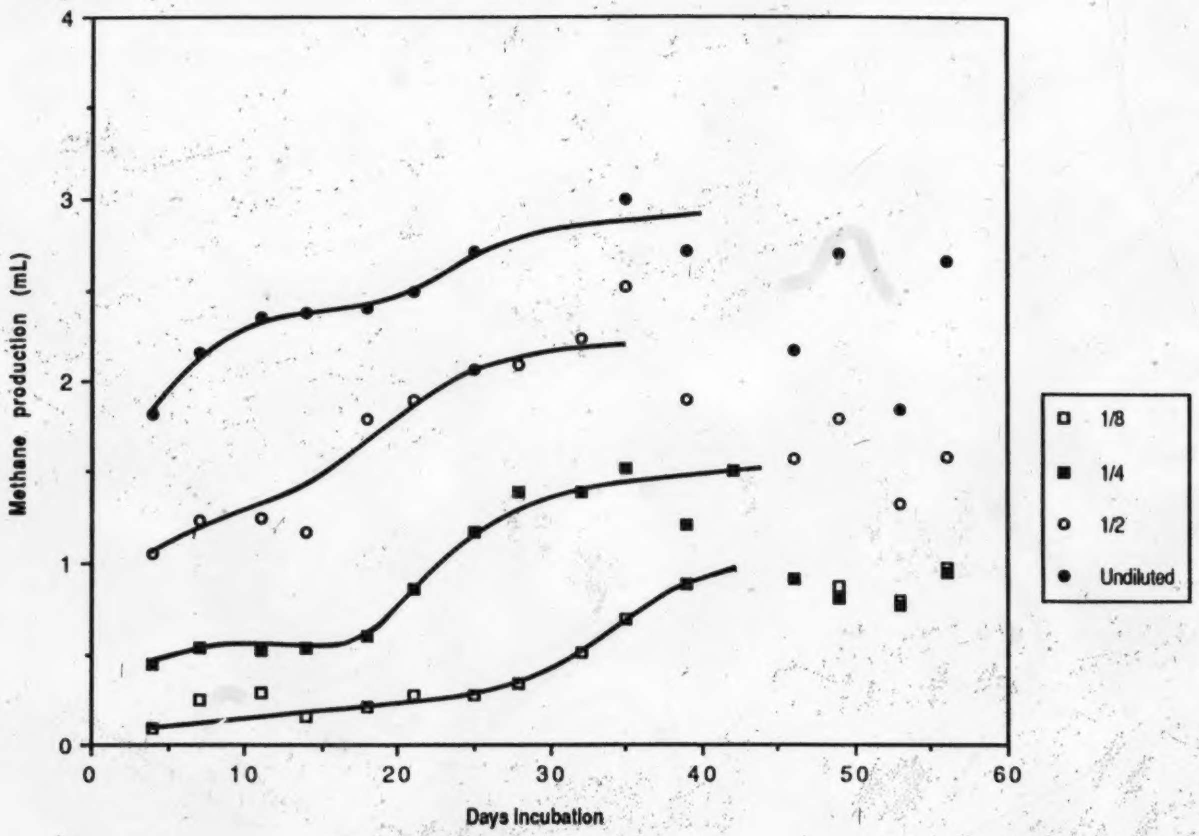

Figure 13. Methane Production from Diluted Sludge Cultures Containing 2-Chlorophenol

The methane came off the column first with a retention time of 4 minutes, and the carbon dioxide was second with a retention time of 8 minutes. Therefore, the two peaks were distinct and resolved enough on the chromatogram so as to be easily distinguishable.

9.8.2. Analysis of the supernatants from enrichment cultures containing radioactive substrates by HPLC and fractionation

Some of these radioactive cultures had their supernatants analyzed by HPLC using solvent I to determine whether or not any intermediates were detectable after $^{14}CO_2$ and $^{14}CH_4$ were detected in the headspace as described in section 9.8.1. The retention times of the known compounds of interest were as follows:

phenol	1.86 minutes
4-CP	2.23 minutes.

This first solvent was based on the solvent used by Realini (1981) in which he used a gradient from 30% to 80% acetonitrile in water with 1% acetic acid.

The first sample analyzed was one of the radioactive 2,4-diCP-containing cultures. A small portion of 0.2 mL was anaerobically removed from the culture and centrifuged for 15 minutes in a clinical centrifuge (g force of 1640). Three 20 µL injections were applied to the column, and 1 fraction per 0.1 minute was collected up to 50 fractions or 5 minutes. Based on the total DPM of 1.1×10^3 applied to the column, only 13.2% of the radioactivity was recovered. 4-CP, an intermediate of 2,4-diCP degradation if the ortho chlorine was initially removed, was not identified in this experiment. Because the major interest was in intermediates of 4-CP degradation, these cultures were not further studied. It should be noted, however, that other enrichment cultures were prepared with non-radioactive 2,4-diCP as the substrate, and after a period of time, 4-CP was the only intermediate detected in the culture by the HP 5890 GC.

9.8.2.1. Analysis of 4-chlorophenol A supernatant by HPLC and fractionation I

Next, one of the cultures containing radioactive 4-CP was analyzed. This particular culture was arbitrarily designated 4-CP A. A 0.3 mL portion was anaerobically removed and centrifuged for 15 minutes in a clinical centrifuge (g force of 1640). Four 30 µL injections were applied with one 0.1 mL fraction collected per 0.1 up to 50 fractions. In this case, of the total radioactivity applied, only 72% of the radioactivity was recovered. The total radioactivity applied was 7 x 10^2 DPM. The radioactivity chromatogram of this sample is shown in Figure 14. As can be seen, the peak containing fractions 28 to 33 contained 49% of the radioactivity added corresponding to 59% of the total radioactivity recovered. Thus, even though this culture displayed mineralization products, mineralization was not complete because the radioactive peak was 4-CP as determined in section 9.8.2.3.

9.8.2.2. Analysis of 4-chlorophenol A supernatant by HPLC and fractionation II

Since the first attempt to analyze sample 4-CP A resulted in only 83% recovery, the supernatant was again applied to determine

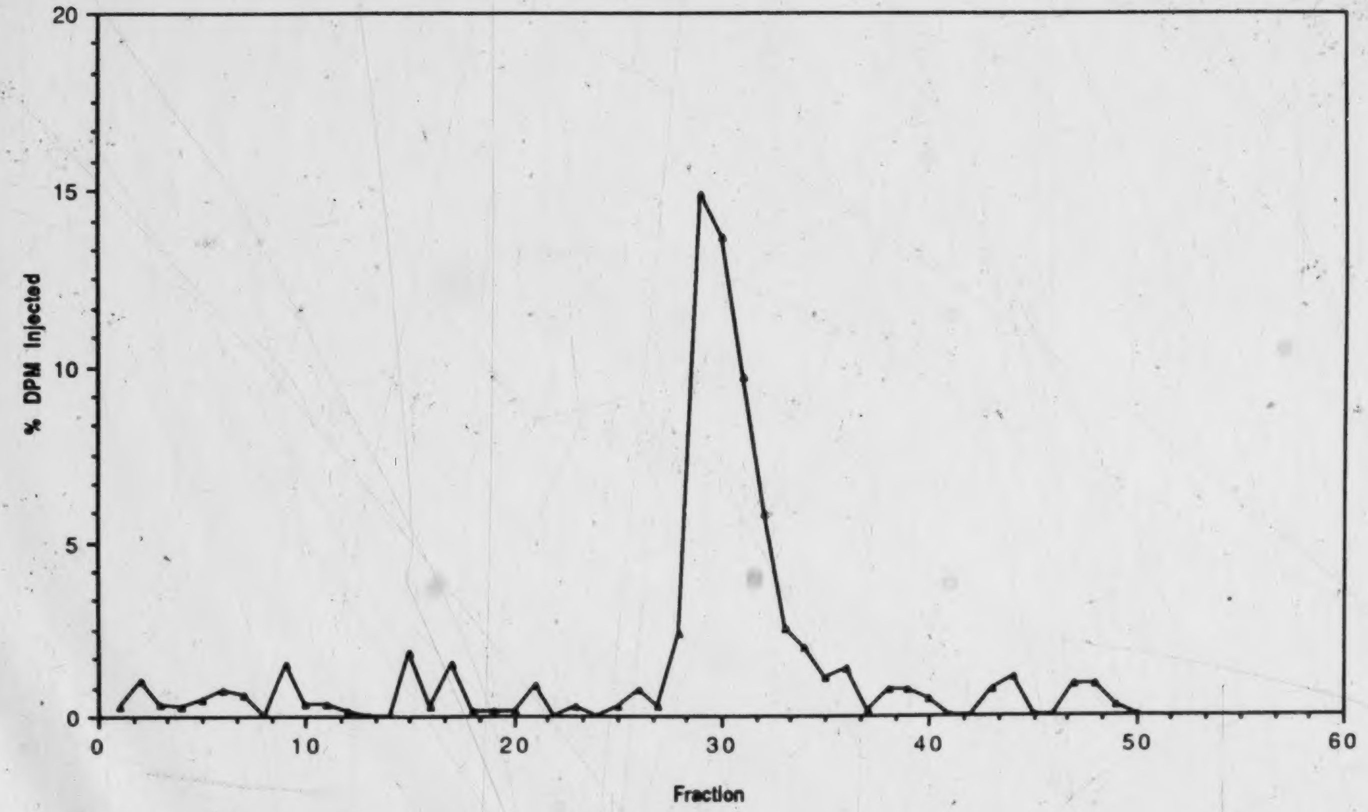

Figure 14. Analysis of 4-CP A Supernatant by HPLC and Fractionation I
(Volume of each fraction = 0.1 mL; 0.1 mL = 0.1 min)
(Retention time of major peak (4-CP) = (Fraction) 29 x 0.1 min = 2.9 min)

whether a better recovery could be obtained. The recovery for this analysis was 102% with a total radioactivity of 7 x 10^2 DPM applied to the column as 4 x 30 μL injections with one 0.1 mL fraction collected per 0.1 minute up to 50 fractions. The chromatogram is shown in Figure 15. The radioactive peak was located in fractions 28 to 35, which was 61% of the 102% recovered, approximately 60% of the total radioactivity, as in the first analysis. Again, the peak was located in the same area as in the first analysis.

9.8.2.3. Analysis of standard 4-chlorophenol by HPLC and fractionation

In the previous analyses with 4-CP A, it was assumed that the large peak covering fractions 28 to 33 was ^{14}C-4-CP. To prove this was the case and to be certain of the fractions that contain pure 4-CP, a radioactive 4-CP standard was analyzed. Fractions were collected so that one 0.1 mL fraction was collected per 0.1 minute up to 50 fractions. The standard was predominantly in fractions 28 to 32. According to Figure 16, the peak covered fractions 27 to 36, corresponding to 95% of the added radioactivity. Thus, the peaks in the previous two analyses were 4-CP. The total radioactivity added in this analysis was 2.2 x 10^4 DPM as 3 x 7 μL injections with a recovery of 97%.

9.8.2.4. Analysis of 4-chlorophenol A supernatant plus standard 4-chlorophenol by HPLC and fractionation I

The 4-CP A sample and 4-CP standard were combined and added to the column to determine whether there was any difference in peak characteristics within the sample. Fractions were collected

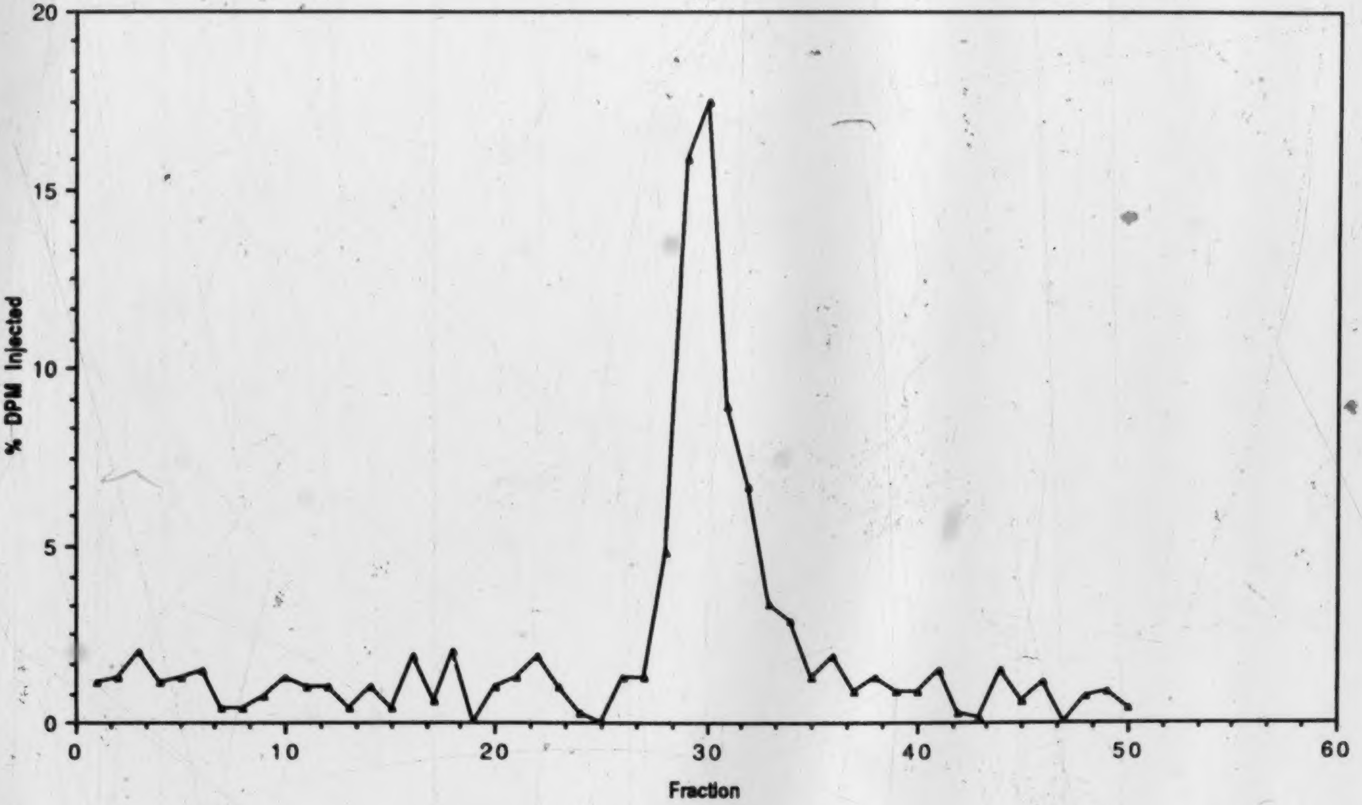

Figure 15. Analysis of 4-CP A Supernatant by HPLC and Fractionation II
(Volume of each fraction = 0.1 mL; 0.1 mL = 0.1 min)
(Retention time of major peak (4-CP) = (Fraction) 30 x 0.1 min = 3 min)

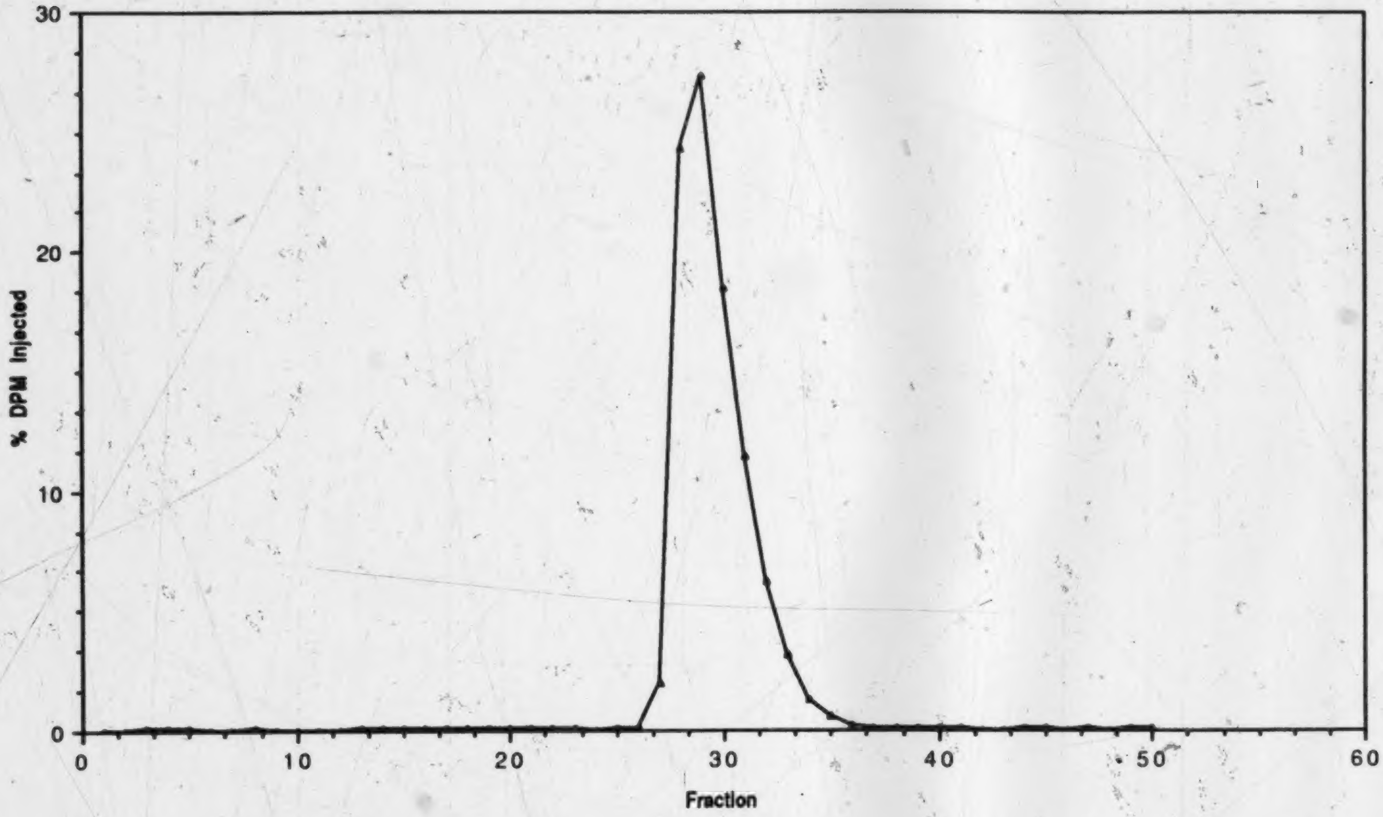

Figure 16. Analysis of Standard 4-CP by HPLC and Fractionation
(Volume of each fraction = 0.1 mL; 0.1 mL = 0.1 min)
(Retention time of major peak (4-CP) = (Fraction) 29 x 0.1 min = 2.9 min)

so that one 0.1 mL fraction was collected per 0.1 minute up to 50 fractions. Most of the radioactivity was clustered from fractions 28 to 36. Figure 17 displayed this same trend. The total radioactivity applied was 4.4 x 10^3 DPM as 4 x 30 µL injections with a recovery of 94%. Fractions 28 to 36 covered 89% of the applied radioactivity.

9.8.2.5. Analysis of 4-chlorophenol A supernatant by HPLC and fractionation III

4-CP sample A was analyzed again, but in this case 60 fractions were collected with 0.5 mL fraction collected per 0.5 minutes. This was done to determine whether there were other peaks that eluted from the column later than 5 minutes, the maximum time the previous analyses using this sample eluted. Most of the radioactivity was collected in fractions 5 to 8 seen in Figure 18 corresponding to 12% of the added radioactivity. The total radioactivity applied was 3.1 x 10^2 DPM as 3 x 30 µL injections with a recovery of 33%. Thus, only 36% of the radioactivity was recovered as 4-CP.

9.8.2.6. Analysis of 4-chlorophenol A supernatant plus standard 4-chlorophenol by HPLC and fractionation II

The combined 4-CP A and standard 4-CP was analyzed for 30 minutes rather than 5 minutes as previously. Fractions were collected with 0.5 mL fraction collected per 0.5 minutes. Most of the radioactivity was collected in fractions 5 to 8 with the chromatogram in Figure 19. Fractions 5 to 8 corresponded to 90% of the added radioactivity. The total radioactivity applied was 4.2 x 10^3 DPM as 3 x 30 µL injections with a recovery of 93%.

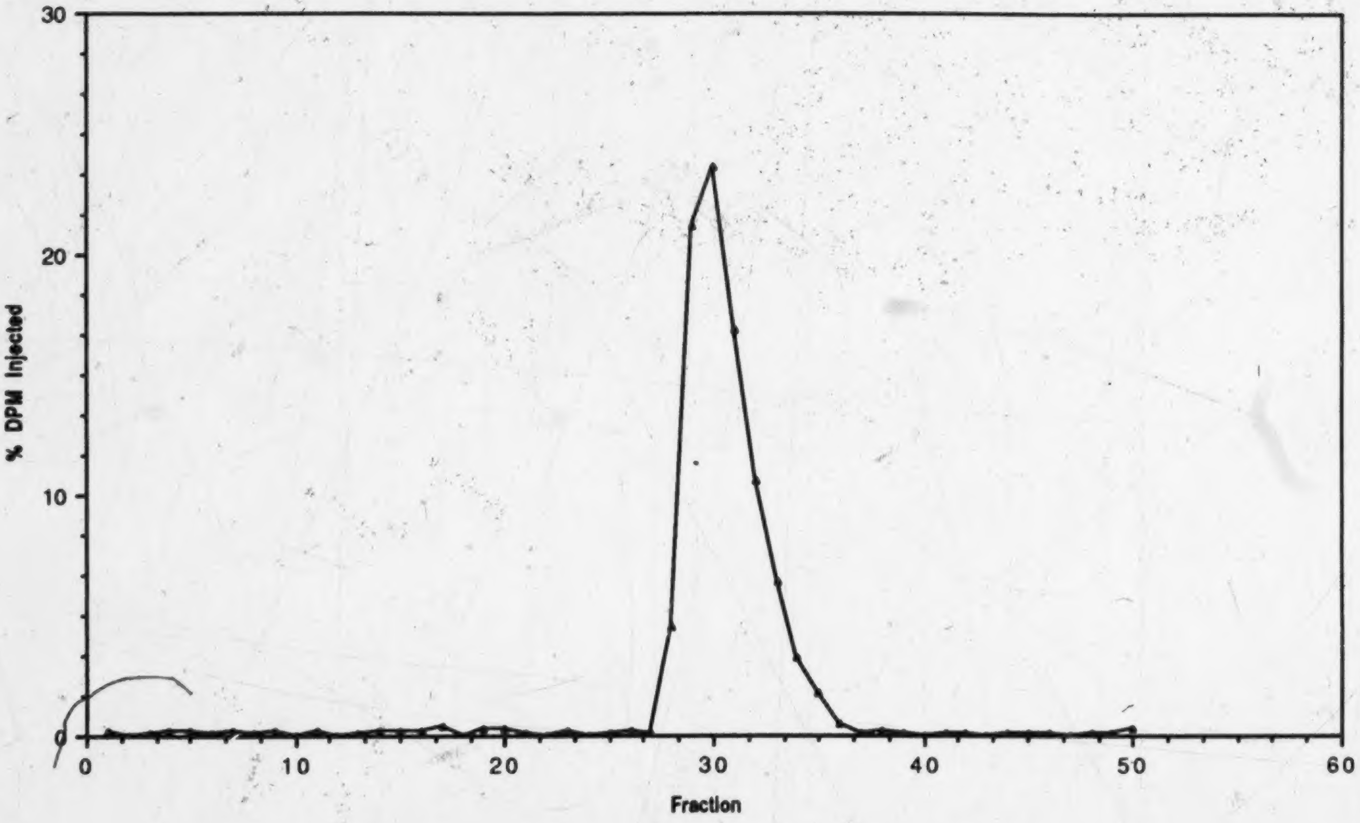

Figure 17. Analysis of 4-CP A Supernatant Plus Standard 4-CP by HPLC and Fractionation I
(Volume of each fraction = 0.1 mL; 0.1 mL = 0.1 min)
(Retention time of major peak (4-CP) = (Fraction) 30 x 0.1 min = 3 min)

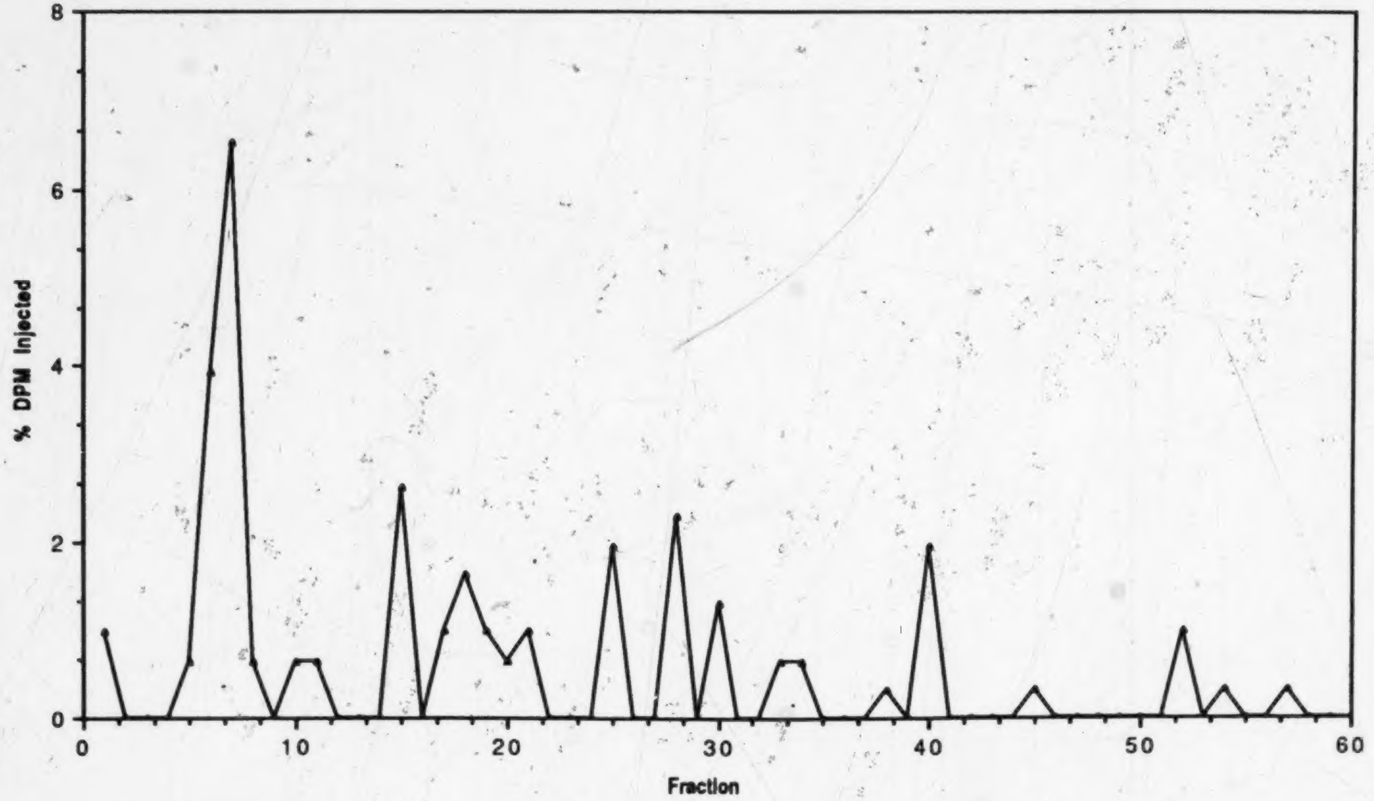

Figure 18. Analysis of 4-CP A Supernatant by HPLC and Fractionation III
(Volume of each fraction = 0.5 mL; 0.5 mL = 0.5 min)
(Retention time of major peak (4-CP) = (Fraction) 7 x 0.5 min = 3.5 min)

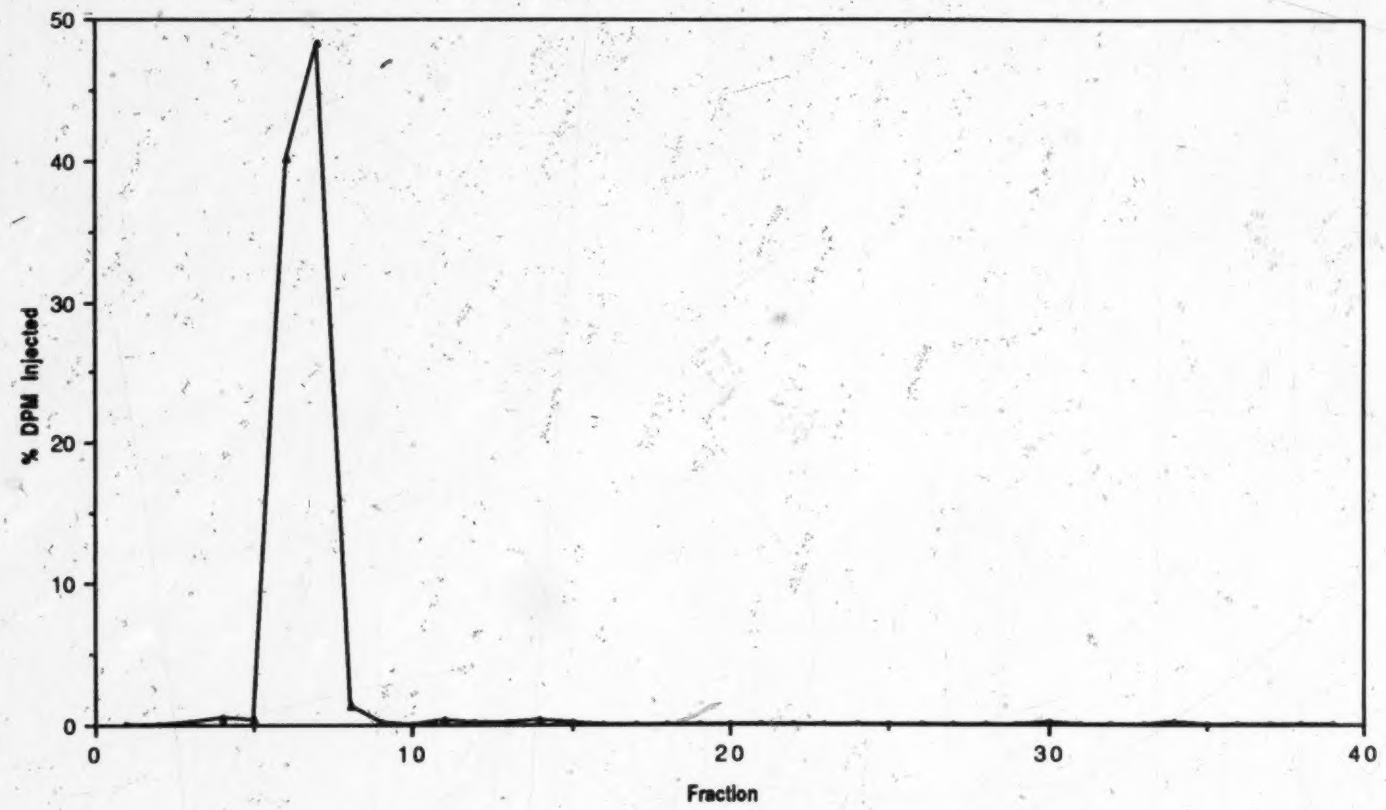

Figure 19. Analysis of 4-CP A Supernatant Plus Standard 4-CP by HPLC and Fractionation II
(Volume of each fraction = 0.5 mL; 0.5 mL = 0.5 min)
(Retention time of major peak (4-CP) = (Fraction) 7 x 0.5 min = 3.5 min)

9.9. Degradation rate of 4-chlorophenol using non-radioactive enrichment cultures as the inoculum

The August, 1987 enrichment cultures were used as inoculum to determine the degradation rate of 4-CP. This was done as soon as it was determined that these enrichment cultures had completely degraded the initial 4-CP added.

9.9.1. Degradation rate of 4-chlorophenol-containing culture F

Prior to preparing any major experiments using this inoculum, a preliminary study was prepared using culture F. Culture F was one of the twenty August enrichment cultures discussed in section 9.3., and this experiment was designed to determine how active the culture was so that it could be used as an inoculum. The letter F was an arbitrary designation. Two 60 mL serum bottles were set up and each contained the following: 5 mL inoculum from a selected enrichment culture, 3.96 mL growth medium, 0.04 mL $Na_2S \cdot 9H_2O$, and 1.0 mL substrate to a final concentration of 40 mg/L 4-CP. One culture had radioactive 4-CP as well. This was done so that the non-radioactive culture could be used to monitor the 4-CP concentration, thus the radioactive culture would be relatively untouched.

As seen in Figure 20, degradation was generally uniform. The degradation rate of the first curve, using all points on the plot via linear regression was 1.8 mg/L/day. By the same method, the degradation rate of the second curve was also 1.8 mg/L/day.

9.9.2. Degradation rate of 4-chlorophenol-containing culture G

After culture F was evaluated, three cultures designated G, H,

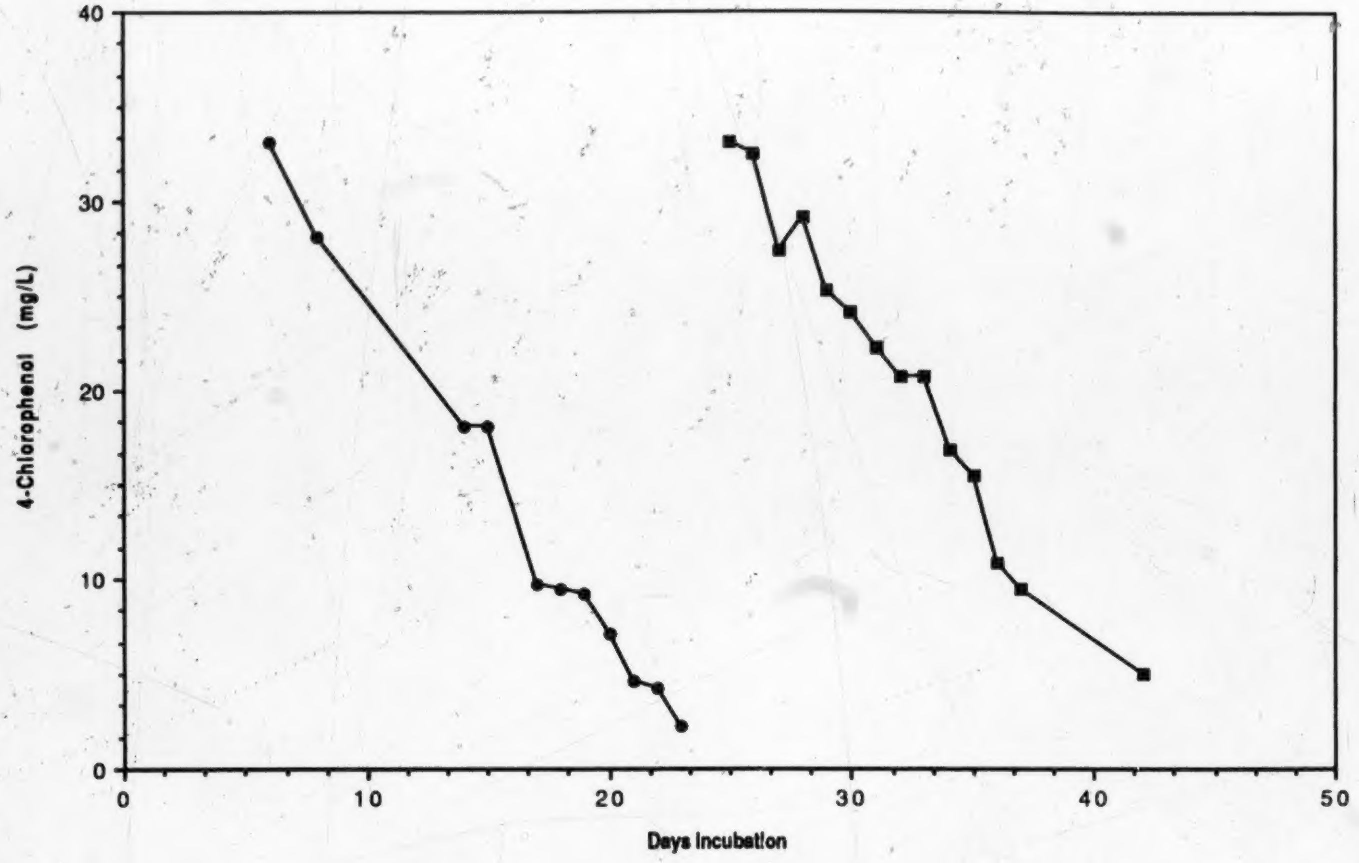

Figure 20. Degradation of 4-Chlorophenol by Culture F

and J were prepared as above with 4-CP to a final concentration of 40 mg/L using culture F as the inoculum. These were to incubate until the culture concentration was below 5 mg/L. After this, more 4-CP was added to the same final concentration of 40 mg/L. In addition, ^{14}C-4-CP was added to each culture when the concentration of 4-CP was below 5 mg/L, then each culture was sacrificed at different times during degradation.

Figure 21 shows the degradation curve of culture G. The rate as determined through linear regression was 1.8 mg/L/day for the first curve using all points. The second curve on this same plot was the result after 4-CP was again added to the culture. The degradation rate for this second curve was 3.9 mg/L/day.

9.9.3. Degradation rate of 4-chlorophenol-containing culture H

Culture H also degraded the substrate much more quickly after the second addition of substrate as shown in Figure 22. The initial degradation rate was 1.0 mg/L/day as determined using all points. An additional amount of 4-CP was added on day 34. The concentration of 4-CP decreased by 6.9 mg/L after one day incubation.

9.9.4. Degradation rate of 4-chlorophenol-containing culture J

Figure 23 displays the degradation curve of culture J. The initial rate calculated using all points for the first addition of substrate was 1.0 mg/L/day. On day 26, additional 4-CP was added to the culture. The degradation rate of 4-CP in 4 days was 3.6 mg/L/day.

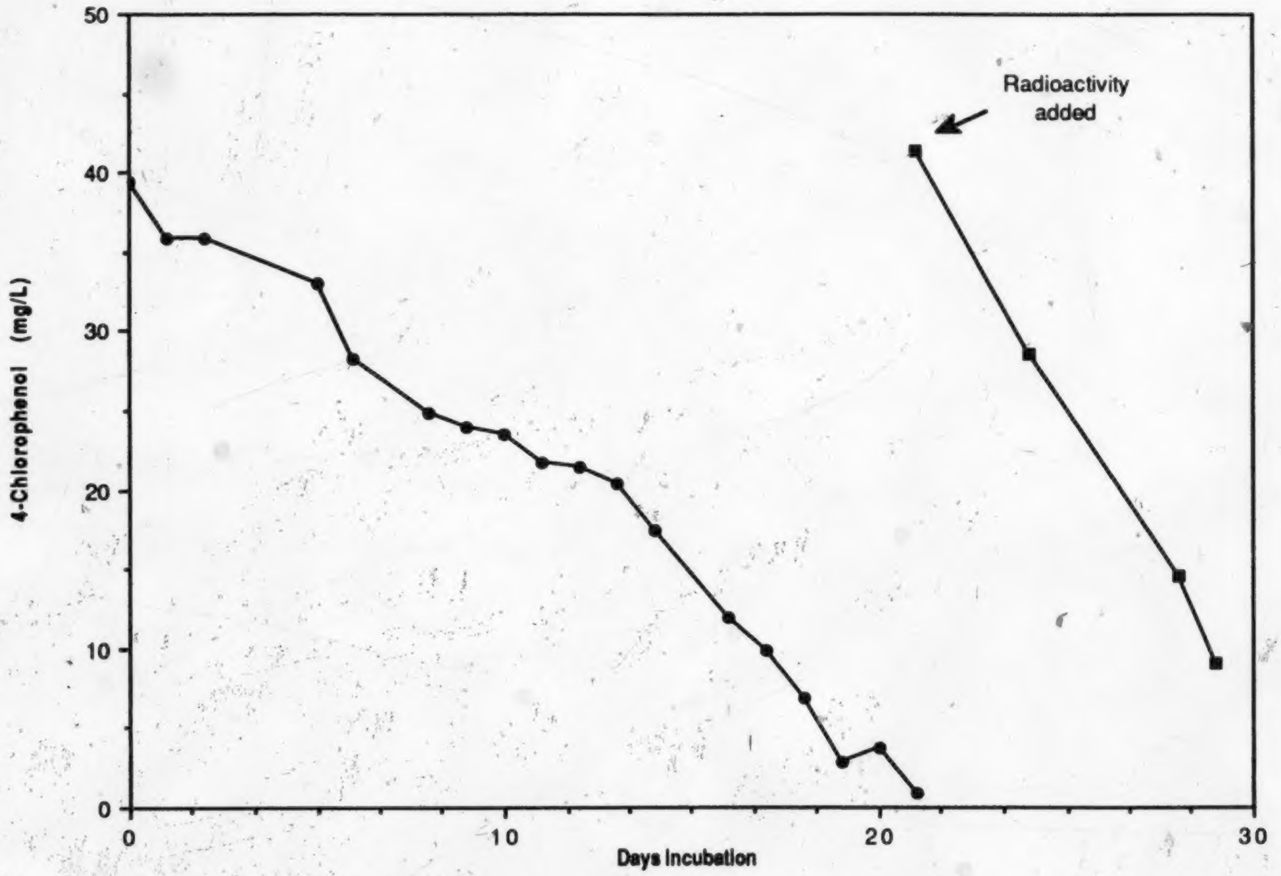

Figure 21. Degradation of 4-Chlorophenol by Culture G

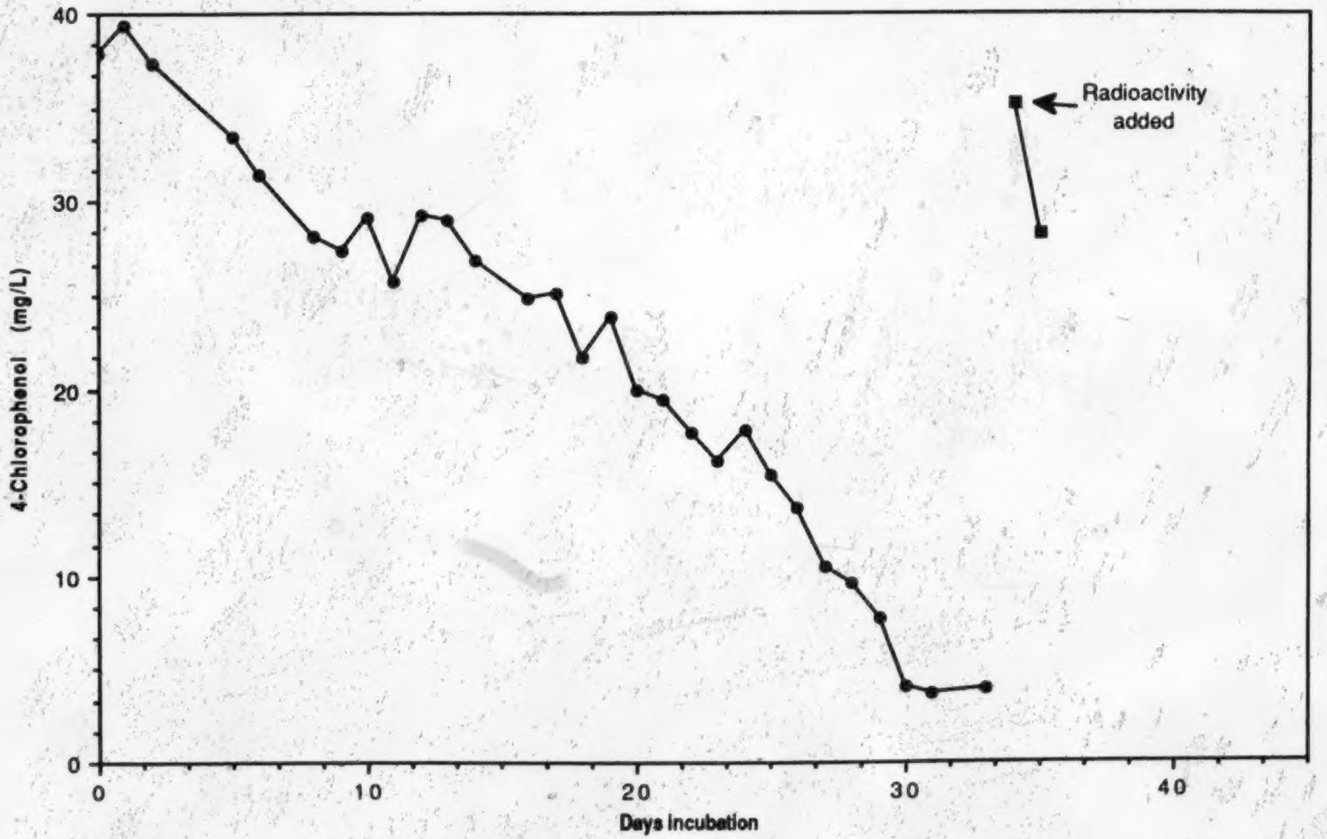

Figure 22. Degradation of 4-Chlorophenol by Culture H

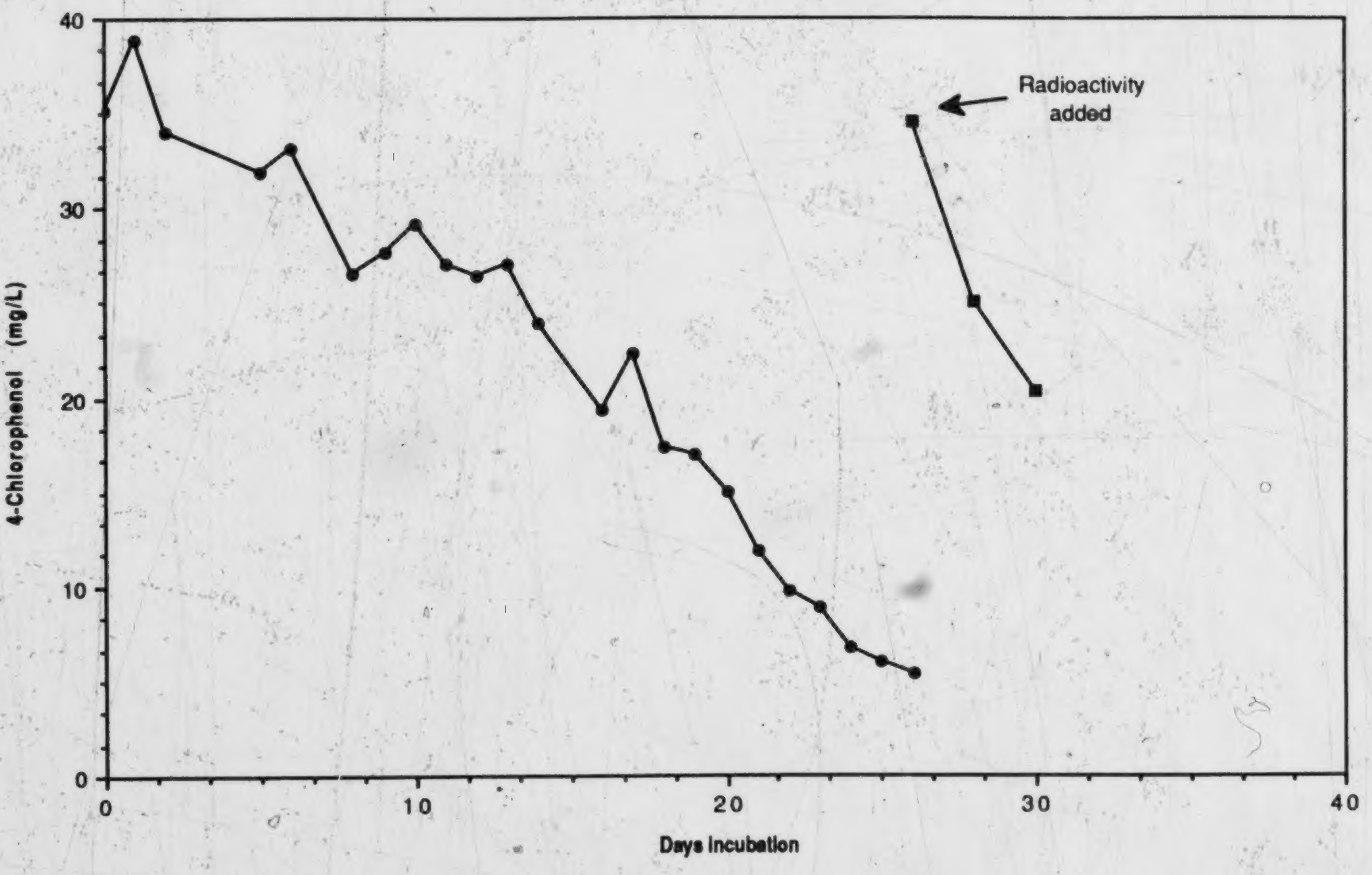

Figure 23. Degradation of 4-Chlorophenol by Culture J

9.10. Analysis of ^{14}C-4-chlorophenol-containing cultures for $^{14}CO_2$, $^{14}CH_4$, and intermediates of 4-chlorophenol degradation

9.10.1. Total radioactivity recovery of culture 4-chlorophenol C: Preliminary

One of the 5 radioactive 4-CP-containing cultures was analyzed for radioactivity in order to observe the distribution of radioactivity. The methane was analyzed by GPC as described in section 8.2.4.1. of Methods and Materials. However, the amount of carbon dioxide in the headspace was not determined. Instead, 8 mL of 4 N NaOH was added so that the final pH was between 12 and 14 to trap the carbon dioxide in solution. The addition of 4 N NaOH resulted in a final pH of 13.

To determine the amount of radioactive carbon dioxide, the culture was left at pH 13 at least overnight to be certain the carbon dioxide was completely within the solution. Next, 10 mL of 6.2 M HCl was added to the culture to a final pH of 1. The culture was flushed for 20 minutes, but some fluor and Carbosorb entered the flushing lines. The culture was left overnight and was again flushed the next day for 20 minutes.

The amount of radioactivity in the supernatant was determined by removing 3 x 3 mL samples from the flushed culture and centrifuging at maximum speed in a clinical centrifuge (g force of 1640) for 10 minutes. If the solids were not completely settled after 10 minutes, the solution was centrifuged for another 10 minutes. Three 600 µL portions were removed with an Eppendorf pipet and counted in an LSC.

The solids were analyzed by removing as much of the remaining liquid as possible from the test tubes. As little water as possible was used to remove the solids quantitatively. The solids were then counted in an LSC. Table 9 shows a summary of the percent recovery of the radioactive label.

9.10.2. Total radioactivity recovery of culture 4-chlorophenol D: Second procedure

Because these enrichment cultures had a 50 mL volume, flushing may not have quantitatively removed the radioactive carbon dioxide because of both the high liquid and solid content. The determinations of headspace volume and analysis of methane, supernatant, and solids were carried out as in the preliminary procedure.

To determine the amount of radioactive carbon dioxide in the culture, 8 mL of 6 N NaOH was added to the culture and left overnight. A 5 mL sample was removed from the culture and placed in a 38 mL serum bottle. The bottle was stoppered and 2 mL of 6.2 M HCl was added to decrease the pH of the culture to 1 to 2. The 5 mL sample was flushed for 10 minutes. This culture is listed as D_a in Table 9. Another 5 mL sample from the same culture had 1 mL of 6.2 M HCl added to it and was analyzed. This culture is listed as D_b in Table 9. D_a and D_b were duplicate analyses of culture 4-CP D. One mL instead of 2 mL of 6.2 M HCl was added to sample D_b to lower the amount of acid added to the culture because 1 mL of acid was enough to lower the pH of the culture to 1 to 2.

Table 9. Initial Radioactivity Recovery of ^{14}C-4-Chlorophenol-Degrading Enrichment Cultures

	Culture		
	C	D_a	D_b
% $^{14}CO_2$ Total	37.0	43.0	37.7
% $^{14}CH_4$	19.0	12.6	12.6
%$^{14}CH_4$/%Total CO_2	0.51	0.29	0.33
% ^{14}C in Liquid	1.90	ND	1.12
% ^{14}C in Solids	0.31	ND	1.04
% Total Recovery	58.2	55.6	52.5

C = 4-CP culture C (GPC Efficiency for this culture was 80%)
D_a = First sample removed from 4-CP culture D (GPC Efficiency for this culture was 88%)
D_b = Second sample removed from 4-CP culture D
ND = Not determined

Five mL of the flushed cultures were removed and centrifuged for 10 minutes. The radioactivity in the supernatant was counted in an LSC as was done previously, and the solids were removed and counted.

9.10.3. Total recovery of radioactivity: Third procedure

The procedure for total recovery of radioactivity was again altered from that previously described in section 9.10.2. In this case, methane and carbon dioxide were analyzed as described in section 8.2.4.1. of Methods and Materials. The initial radioactivity at time 0 was determined as before by centrifuging the flushed culture and removing liquid to be counted in an LSC.

The change in procedure was as follows: after measuring the ^{14}C-carbon dioxide and ^{14}C-methane, 1 mL of 6.2 M HCl was added to the 10 mL radioactive culture. The culture was then flushed for at least 10 minutes. A sample was removed as before by centrifuging the flushed culture and removing liquid to be counted. The solids were analyzed by placing a vacuum filtration apparatus on an Erlenmeyer flask with a sidearm. Three 0.5 mL samples were removed from the flushed culture. Each sample was vacuum filtered and then washed with 3 x 5 mL of distilled water. The filter was removed and counted in 10 mL of ACS fluor.

This method was applied to cultures K, G, H, and J as described below, and the results are summarized in Table 10. In Table 10 as well as subsequent tables containing total radioactivity recovery

Table 10. Total Recovery of Radioactivity From ^{14}C-4-Chlorophenol-Containing Cultures

		Culture K[1]	Culture G[2]	Culture H[3]	Culture J[2]
% $^{14}CO_2$	Gas	15.6	8.60	1.88	6.89
	Liquid	23.5	23.5	4.15	9.69
	Total	39.1	32.1	6.03	16.6
% $^{14}CH_4$		25.8	21.5	4.04	28.0
% CH_4/%Total CO_2		0.66	0.67	0.67	1.69
% ^{14}C in Liquid		5.55	12.7	47.9	29.4
% ^{14}C in Solids		1.84	3.04	7.59	5.75
% Total Recovery*		72.3	69.3	65.6	79.8

* Total Recovery = % Total CO_2 + % $^{14}CH_4$ + ^{14}C in Liquid + ^{14}C in Solids

[1] GPC Efficiency was 94%

[2] GPC Efficiency was 69%

[3] GPC Efficiency was 100%

data, the percentages were calculated as follows:

$$\frac{\text{Radioactivity (DPM) recovered as } {}^{14}\text{C}}{\text{Radioactivity (DPM) at time 0}} (100)$$

The methane to carbon dioxide ratio was determined because the stoichiometry of 4-CP biodegradation was studied and compared to the expected percent methane and carbon dioxide values in Appendix F.

9.10.3.1. Total radioactivity recovery in ^{14}C-4-CP-containing culture K

Culture K was prepared at the same time as culture F, except that K had radioactive 4-CP added also. This culture was incubated for a total of 26 days. At the time that this culture was sacrificed, 9.2 mg/L of 4-CP was still present in culture. The initial 4-CP concentration was not determined but the stock was prepared so that the initial concentration was 40 mg/L. Therefore, approximately 77% of the substrate was degraded. The distribution of radioactivity for this culture is shown in Table 10. The amount of radioactivity added at the beginning of incubation was calculated based on the amount of radioactivity in the stock solution rather than directly from culture.

9.10.3.2. Total radioactivity recovery in ^{14}C-4-CP-containing culture G

Culture G was incubated for 9 days after the addition of ^{14}C-4-CP, and 74% of the substrate was depleted at the end of this period. As can be seen in Table 10, nearly the same amount of total ^{14}C-carbon dioxide and ^{14}C-methane was produced from almost the

6

same degree of degradation as that of culture K. Also, the %$^{14}CH_4$/% total $^{14}CO_2$ ratios were virtually identical. Cultures G, H, and J had the initial radioactivity analyzed by counting 3 x 10 µL samples of the radioactive cultures at the time that the radioactivity was added (time 0).

9.10.3.3. Total radioactivity recovery in ^{14}C-4-CP-containing culture H

Culture H was incubated for 2 days after the addition of the ^{14}C-4-CP. In this culture, the highest concentration of radioactivity was in the supernatant, whereas the radioactive carbon dioxide and methane made up only 10% of the total radioactivity (Table 10).

9.10.3.4. Total radioactivity recovery in ^{14}C-4-CP-containing culture J

Culture J incubated for 4 days after adding the ^{14}C-4-CP. Although this culture degraded only 6% more substrate than culture H, gas production was much higher (Table 10). Average recovery for all cultures in Table 10 was 71.8% ± 6.0%.

9.10.4. Total radioactivity recovery from acidified culture H

Because culture H had the lowest recovery of the four cultures analyzed, the acidified culture was again analyzed to determine where any losses may have occurred during recovery.

The frozen sample was thawed at room temperature, and 3 x 10 µL samples of the culture were placed in scintillation vials to which 10 mL of ACS fluor was added. They were counted in an LSC, with the total radioactivity of the culture equivalent to 100%. Note that 100% radioactivity in this culture was based on the total radioactivity in the culture suspension after acidification. However, 100% radioactivity in the rest of the cultures analyzed for total radioactivity was based on the total radioactivity in culture suspension at time 0 (time of radioactive substrate addition).

The culture was diluted by adding 1 mL of the culture to 4 mL of distilled water which was previously placed in a stoppered 38 mL serum bottle. This suspension was flushed for 10 minutes to determine whether ^{14}C-carbon dioxide was still present.

To determine the radioactivity in the solids, 1 mL of the diluted culture was filtered through a Swinex filter containing a 0.45 µm pore diameter filter. The filtrate was collected in a scintillation vial and 10 mL ACS fluor was added. The filter was then washed with 3 x 1 mL of distilled water with each wash collected in a separate scintillation vial. The filter was then carefully removed with tweezers, placed in a scintillation vial, 10 mL of ACS fluor was added, and the filter was counted. This was carried out 3 times so that 3 filters were counted per sample. Thus, as seen in Table 11, it appeared that previous losses occurred as the filters were washed because the previous recoveries did not involve recovering the washes.

Both dry and wet (washed with 3 x 1 mL distilled water) filters were counted in an LSC to determine whether they were adding any extra counts to the background value. As a result, the values for the filters were the same as the background. Thus, there was no interference from the filters.

Table 11. Total Recovery of Radioactivity From Acidified Culture H*

	% Radioactivity
CO_2	0.39
Filters (Solids)	26.4
Filtrate	36.0
Wash 1	22.8
Wash 2	10.3
Wash 3	4.14
Supernatant	10.4
Solids + Filtrate + Washes	99.6
Solids + Filtrate + Washes + CO_2	100

* The basis for percent recovery of radioactivity is different than the rest of the radioactivity recovery tables as discussed in section 9.10.4.

9.11. Determination of whether solids interfered with the counting efficiency of the liquid scintillation counter for the third radioactivity recovery procedure

The first and second radioactivity recovery procedures described in sections 9.10.1. and 9.10.2. resulted in low counting efficiencies such as 35% and 41% due to the large amount of solids present. The procedure described in section 9.10.4. did not appear to interfere with the counting efficiency. However, a test was carried out to prove that the solids would not interfere with radioactive counts.

Phenol was chosen as a source of radioactivity to test for this. Three 2 µL samples of the radioactive phenol were placed in scintillation vials to which 10 mL of ACS fluor was added, and were counted in an LSC. Next, 3 scintillation vials containing 0.5 mL of filtered sludge each had 2 µL of the radioactive phenol added, then 10 mL of ACS fluor, and were also counted.

A loss of approximately 10% in counting efficiency of the LSC was noted. This corresponded to a difference of 28 DPM between the vial containing only the ^{14}C-phenol and the vial containing the ^{14}C-phenol plus solids. This was thus considered negligible interference.

9.12. Analysis of ^{14}C-4-chlorophenol-containing cultures by HPLC and fractionation

The solvent used previously to analyze the radioactivity in the 4-CP-containing enrichment cultures was used to analyze the supernatants of cultures G, H, and J. This was done to search for intermediates because these cultures were acidified before the 4-CP was completely degraded. The next two solvents described in section 8.2.5.2. of Methods and Materials were used to analyze these cultures.

The retention times of known compounds using solvent II were as follows:

acetate	1.23 minutes
phenol	2.83 minutes
4-CP	5.53 minutes.

The retention times of known compounds using solvent III were as follows:

acetate	2.83 minutes
benzoate	4.87 minutes
phenol	7.06 minutes
4-CP	19.65 minutes.

9.12.1. Culture G

9.12.1.1. Analysis of culture G supernatant by HPLC and fractionation: Solvent I

For this culture, as well as cultures H and J, the supernatant was obtained by centrifuging a small portion of the culture for 10 minutes at maximum speed in a clinical centrifuge (g force of 1640). Then the supernatant was removed and filtered through a 0.45 µm pore diameter filter. The resultant filtrate was analyzed.

Culture G was first analyzed on solvent I. This culture was sacrificed after 74% of the substrate was degraded. Fractions 4 to 7 contained 100% of the radioactivity added. The plot of this analysis (Figure 24) shows one large peak with no other significant peaks.

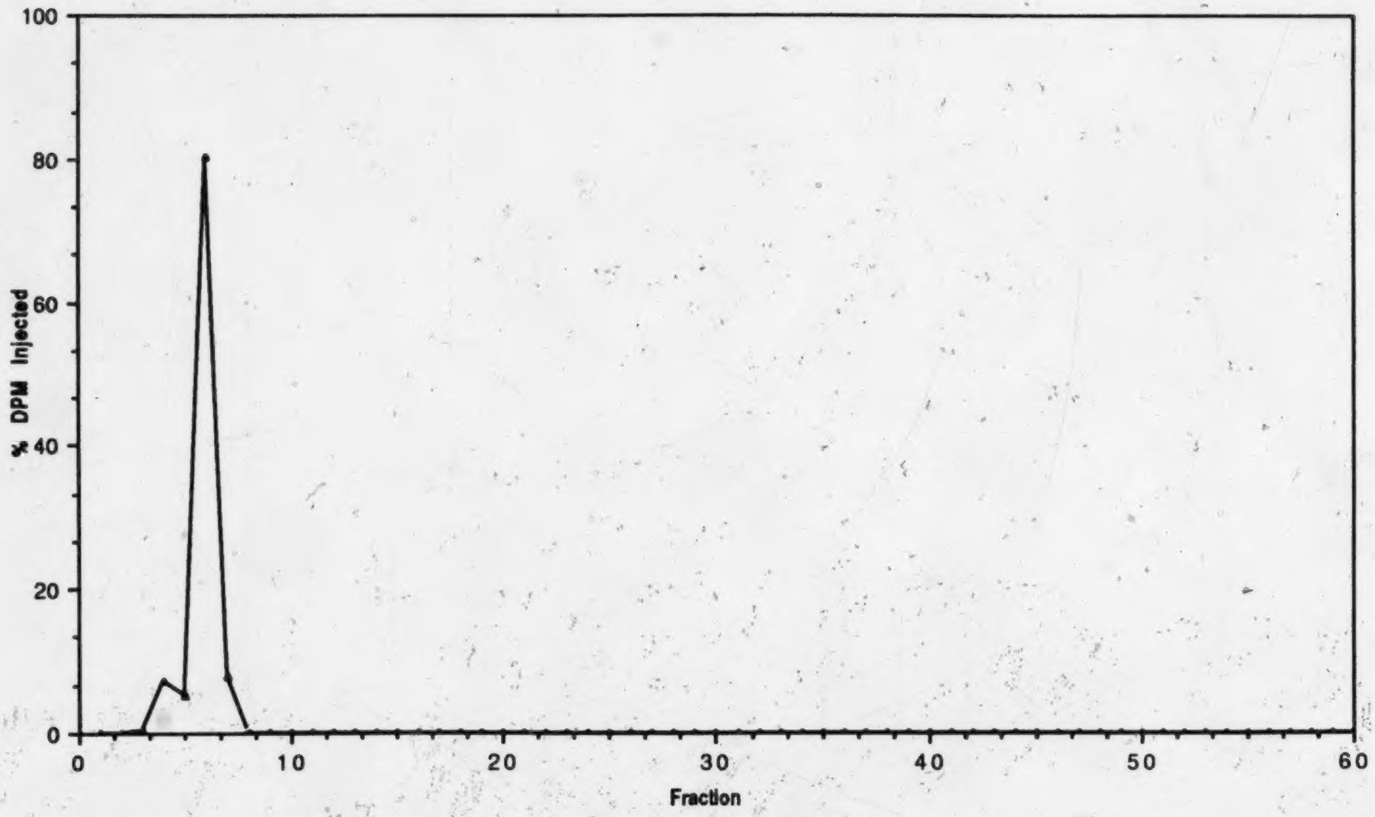

Figure 24. Analysis of Culture G Supernatant by HPLC and Fractionation: Solvent I
(Volume of each fraction = 0.35 mL; 0.35 mL = 0.35 min)
(Retention time of major peak (4-CP) = (Fraction) 6 x 0.35 = 2.1 min)

With 80% of the radioactivity in fraction 5, and comparing this to the retention time of 4-CP, this peak appeared to be 4-CP. Fractions were collected as 0.35 mL per 0.35 minutes up to 60 fractions. The total radioactivity applied was 5.1 x 10^3 DPM as 3 x 50 µL injections. Total radioactivity recovery was 101%.

9.12.1.2. Analysis of culture G supernatant by HPLC and fractionation: Solvent II

Culture G was next analyzed using solvent II. This was done so that 4-CP would elute much later just in case radioactive compounds other than 4-CP were eluting in the first fractions. Compounds eluting earlier could have been obscured by the early elution of 4-CP.

The 4-CP eluted much later and 89% of the radioactivity applied was 4-CP recovered in fractions 16 to 19. Observing the plot (Figure 25), there was a smaller peak prior to 4-CP elution that comprised 8.4% of the radioactivity collected in fractions 5 to 9. It was believed that this was acetate. The total radioactivity applied to the column was 3.0 x 10^3 DPM as 3 x 30 µL injections. Fractions were collected as 0.35 mL per 0.35 minutes up to 70 fractions. Total radioactivity recovery was 110%.

9.12.1.3. Analysis of standard ^{14}C-acetate by HPLC and fractionation: Solvent II

In order to determine whether the radioactive peak seen prior to 4-CP was acetate when culture G supernatant was analyzed via HPLC (Figure 24), authentic radioactive acetate was analyzed by the same method.

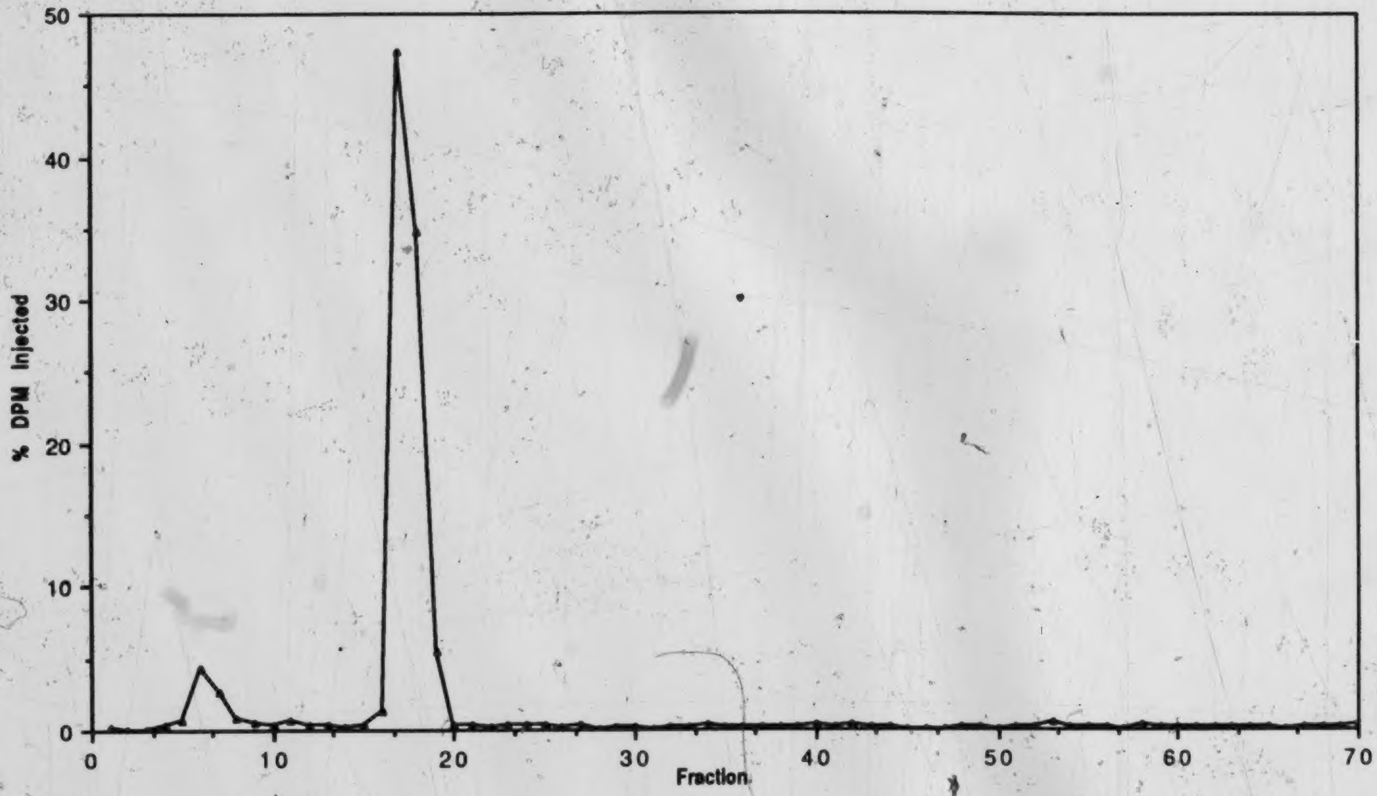

Figure 25. Analysis of Culture G Supernatant by HPLC and Fractionation: Solvent II
(Volume of each fraction = 0.35 mL; 0.35 mL = 0.35 min)
(Retention time of major peak (4-CP) = (Fraction) 17 x 0.35 min = 5.95 min)

The acetate eluted in fractions 5 to 8 resulting in 94% of the applied radioactivity. As a plot in Figure 26, the acetate peak had the same retention time as the small peak in Figure 25, as well as in the same fractions. Thus, this peak was presumably acetate. The total radioactivity applied was 4.8 x 10^2 DPM as 2 x 15 µL injections. Fractions were collected as 0.35 mL per 0.35 minutes up to 20 fractions. Total radioactivity recovery was 111%.

9.12.1.4. Analysis of culture G supernatant by HPLC and fractionation: Solvent III

Culture G was analyzed using solvent III which gave better separation between acetate and 4-CP, in order to detect other peaks that may fall between acetate and 4-CP.

The acetate eluted in fractions 2 to 4 accounting for 6.8% of the radioactivity. The 4-CP eluted much later in fractions 19 to 22 accounting for 89% of the added radioactivity. The plot of this analysis (Figure 27) was similar to culture G analysis on solvent II with a larger gap between the acetate and 4-CP. The total radioactivity applied was 2.0 x 10^3 DPM as one 60 µL injection. Fractions were collected as 1.0 mL per minute up to 40 fractions. Total radioactivity recovery was 101%.

9.12.1.5. Analysis of standard ^{14}C-acetate by HPLC and fractionation: Solvent III

Radioactive acetate was analyzed using this solvent to again prove that the initial peak seen was acetate as assumed.

The acetate eluted in fractions 3 to 7 accounting for 93% of the added radioactivity. In this case, 0.5 mL fractions were

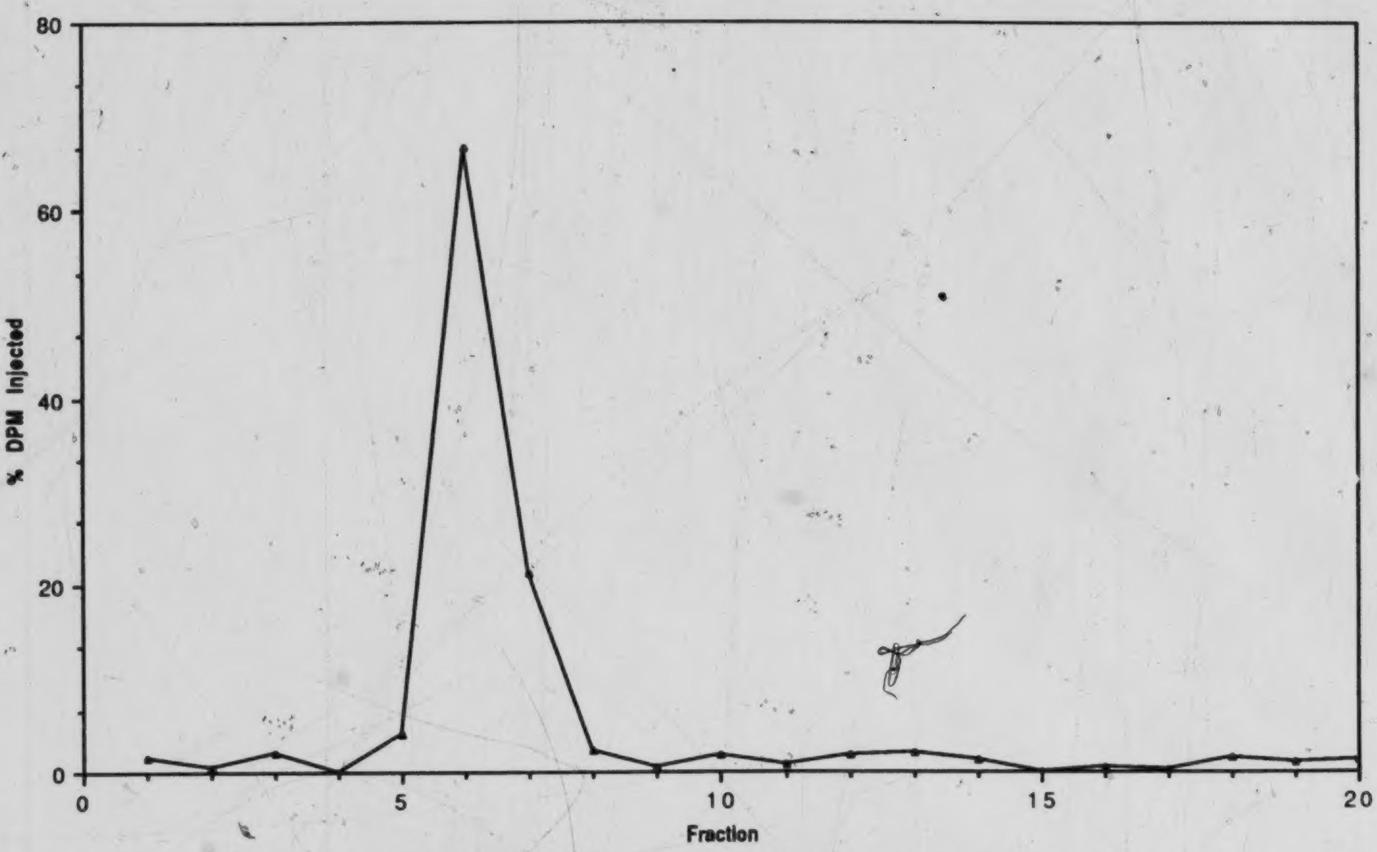

Figure 26. Analysis of Standard Acetate by HPLC and Fractionation: Solvent II
(Volume of each fraction = 0.35 mL; 0.35 mL = 0.35 min)
(Retention time of major peak (acetate) = (Fraction) 6 x 0.35 min = 2.1 min)

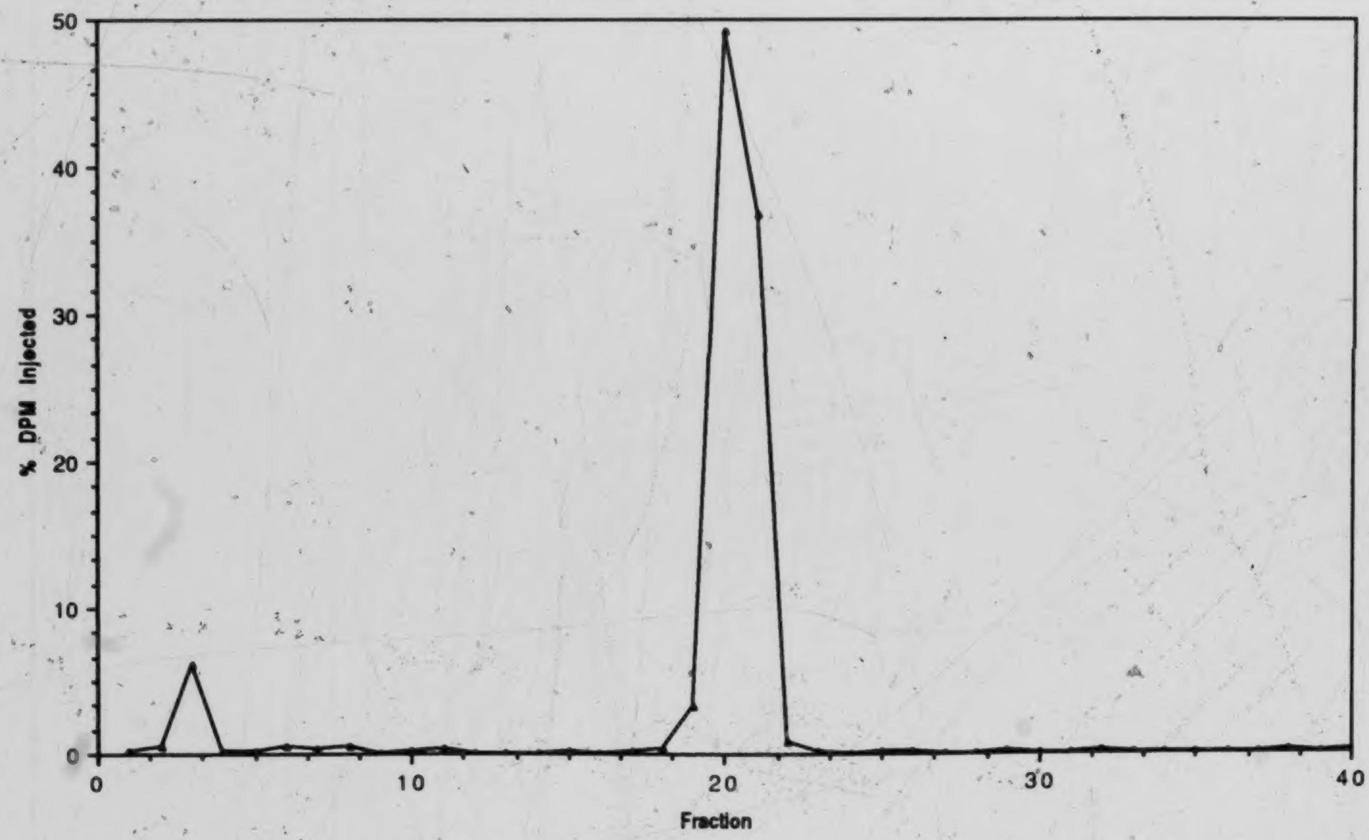

Figure 27. Analysis of Culture G Supernatant by HPLC and Fractionation: Solvent III
(Volume of each fraction = 1 mL; 1 mL = 1 min)
(Retention time of major peak (4-CP) = (Fraction) 20 x 1 min = 20 min)

collected every 0.5 minutes up to 10 fractions. Therefore, the peak (Figure 28) did have the same retention time as the initial peak in Figure 27 and was presumably acetate. The total radioactivity applied was 9.4×10^2 DPM added as 3 x 20 µL injections. Total radioactivity recovery was 98%.

9.12.2. Culture H

9.12.2.1. Analysis of culture H supernatant by HPLC and fractionation: Solvent II

Culture H was sacrificed after 32% of the substrate was degraded. Because this culture had nearly half of the radioactivity in the liquid with low radioactive carbon dioxide and methane production, it was believed that this culture would have detectable intermediates in solution.

After analyzing this culture supernatant by HPLC using solvent II, some acetate was recovered but constituted only 2.3% of the radioactivity in fractions 5 to 8 while 4-CP accounted for 97% of the radioactivity in fractions 16 to 20. The plot of this analysis is shown in Figure 29. The total radioactivity applied was 1.1×10^4 DPM as 3 x 30 µL injections. Fractions were collected as 0.35 mL per 0.35 minutes up to 70 fractions with a total recovery of 103%.

9.12.2.2. Analysis of culture H supernatant by HPLC and fractionation: Solvent III

The supernatant from culture H was also analyzed by HPLC using solvent III. Acetate occurred in fractions 2 to 4, making up only 2.0% of the total radioactivity applied, again, a small portion of

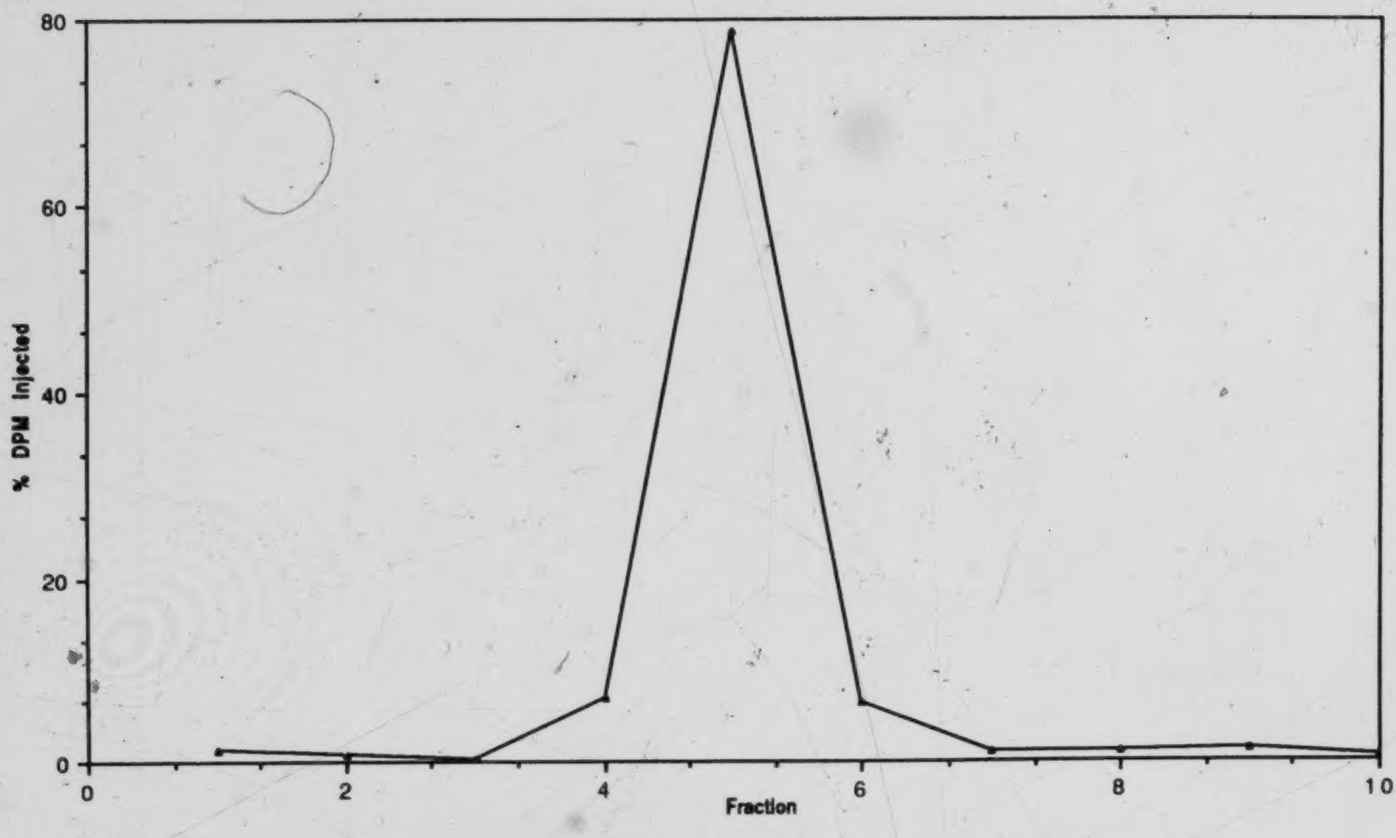

Figure 28. Analysis of Standard Acetate by HPLC and Fractionation: Solvent III
(Volume of each fraction = 0.5 mL; 0.5 mL = 0.5 min)
(Retention time of major peak (acetate) = (Fraction) 5 x 0.5 = 2.5 min)

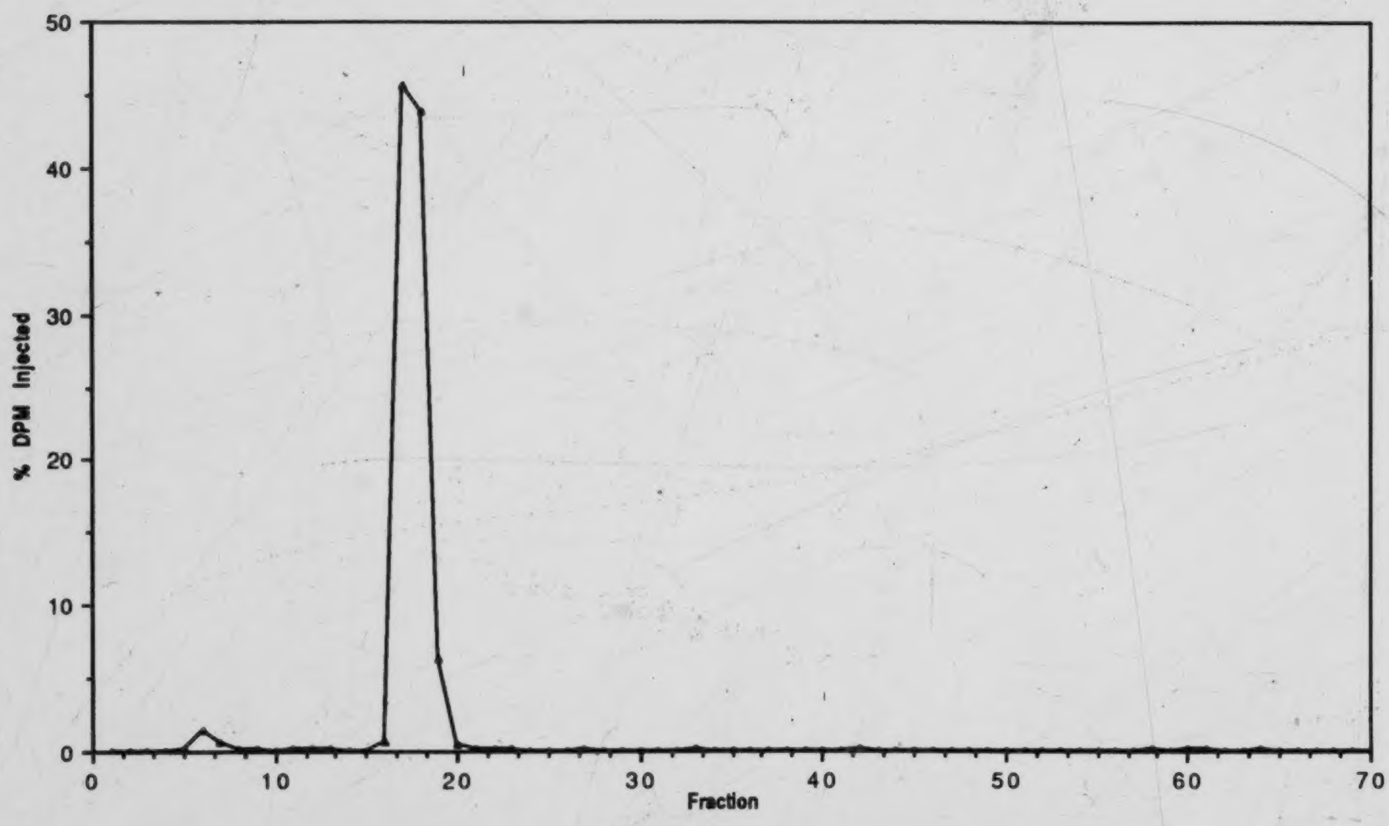

Figure 29. Analysis of Culture H Supernatant by HPLC and Fractionation: Solvent II
(Volume of each fraction = 0.35 mL; 0.35 mL = 0.35 min)
(Retention time of major peak (4-CP) = (Fraction) 17 x 0.35 min = 5.95 min)

the total radioactivity. The 4-CP, however, made up 96% in fractions 19 to 22 also shown in Figure 30. Fractions were collected as 1.0 mL per minute up to 40 fractions. The total radioactivity applied was 7.7 x 10^3 DPM as one 60 µL injection with a total recovery of 99%.

9.12.3. Culture J

9.12.3.1. Analysis of culture J supernatant by HPLC and fractionation: Solvent I

Culture J was sacrificed after 38% of the substrate was depleted. This culture had higher amounts of ^{14}C-carbon dioxide and ^{14}C-methane produced although nearly 30% of the radioactivity was still in solution.

This culture was first analyzed using solvent I. In this case, fractions 4 to 7 contained 99% of the added radioactivity. Figure 31 shows a large peak with a shoulder just before the major peak. The major peak was the 4-CP. Fractions were collected as 0.35 mL per 0.35 minutes up to 60 fractions. The total radioactivity applied was 1.2 x 10^4 DPM as 3 x 50 µL injections with a total recovery of 99%.

9.12.3.2. Analysis of culture J supernatant by HPLC and fractionation: Solvent II

Culture J was also analyzed using solvent II. Acetate was recovered in fractions 5 to 8 as 5.4% of the radioactivity. The 4-CP was recovered in fractions 16 to 20 as 95% of the radioactivity. The acetate peak was very slight in comparison to the larger 4-CP peak (Figure 32). Fractions were collected as 0.35 mL per 0.35 minutes up to 70 fractions. The total radioactivity applied was 6.9 x 10^3 DPM as

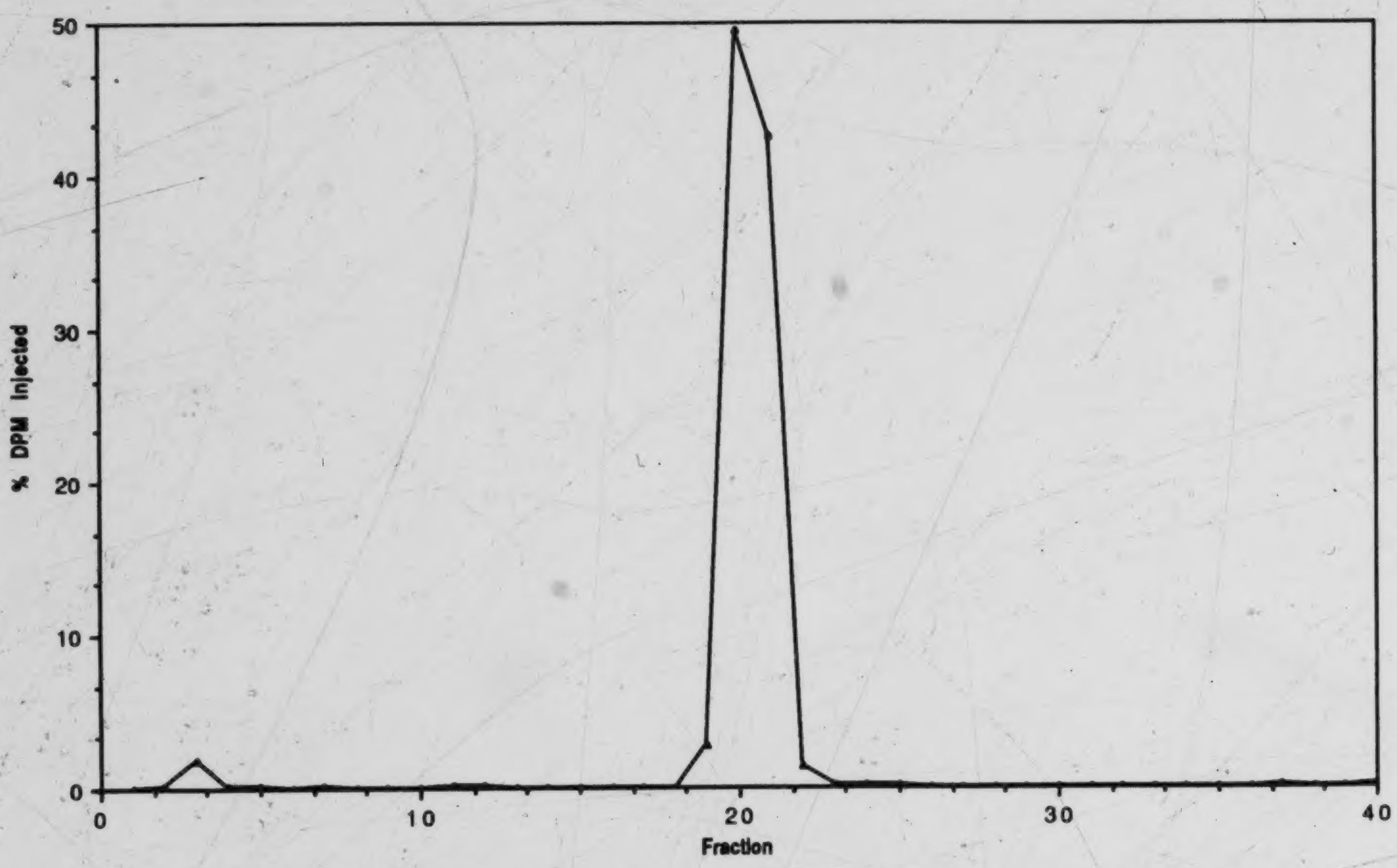

Figure 30. Analysis of Culture H Supernatant by HPLC and Fractionation: Solvent III
(Volume of each fraction = 1 mL; 1 mL = 1 min)
(Retention time of major peak (4-CP) = (Fraction) 20 x 1 min = 20 min)

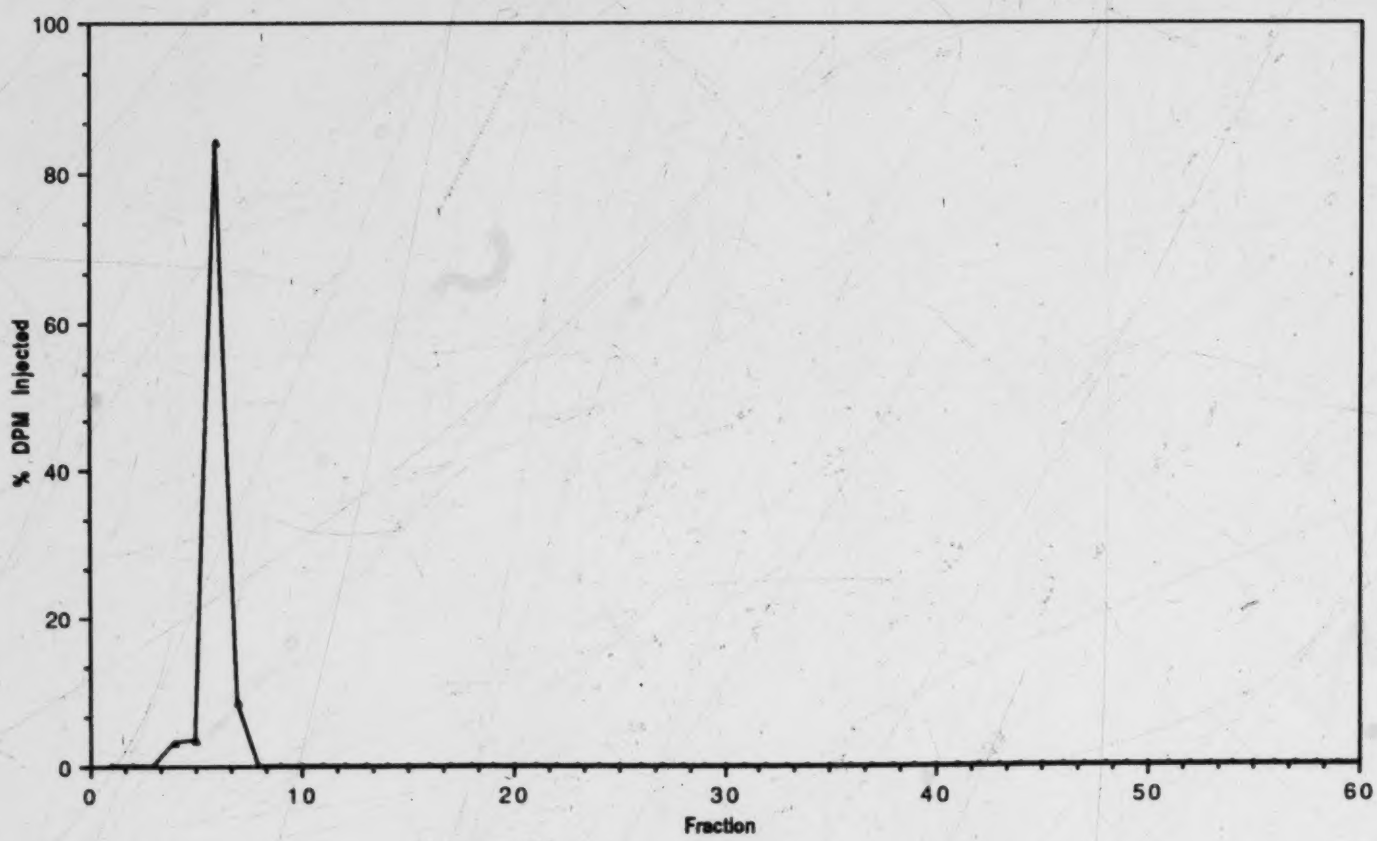

Figure 31. Analysis of Culture J Supernatant by HPLC and Fractionation: Solvent I
(Volume of each fraction = 0.35 mL; 0.35 mL = 0.35 min)
(Retention time of major peak (4-CP) = (Fraction) 6 x 0.35 min = 2.1 min)

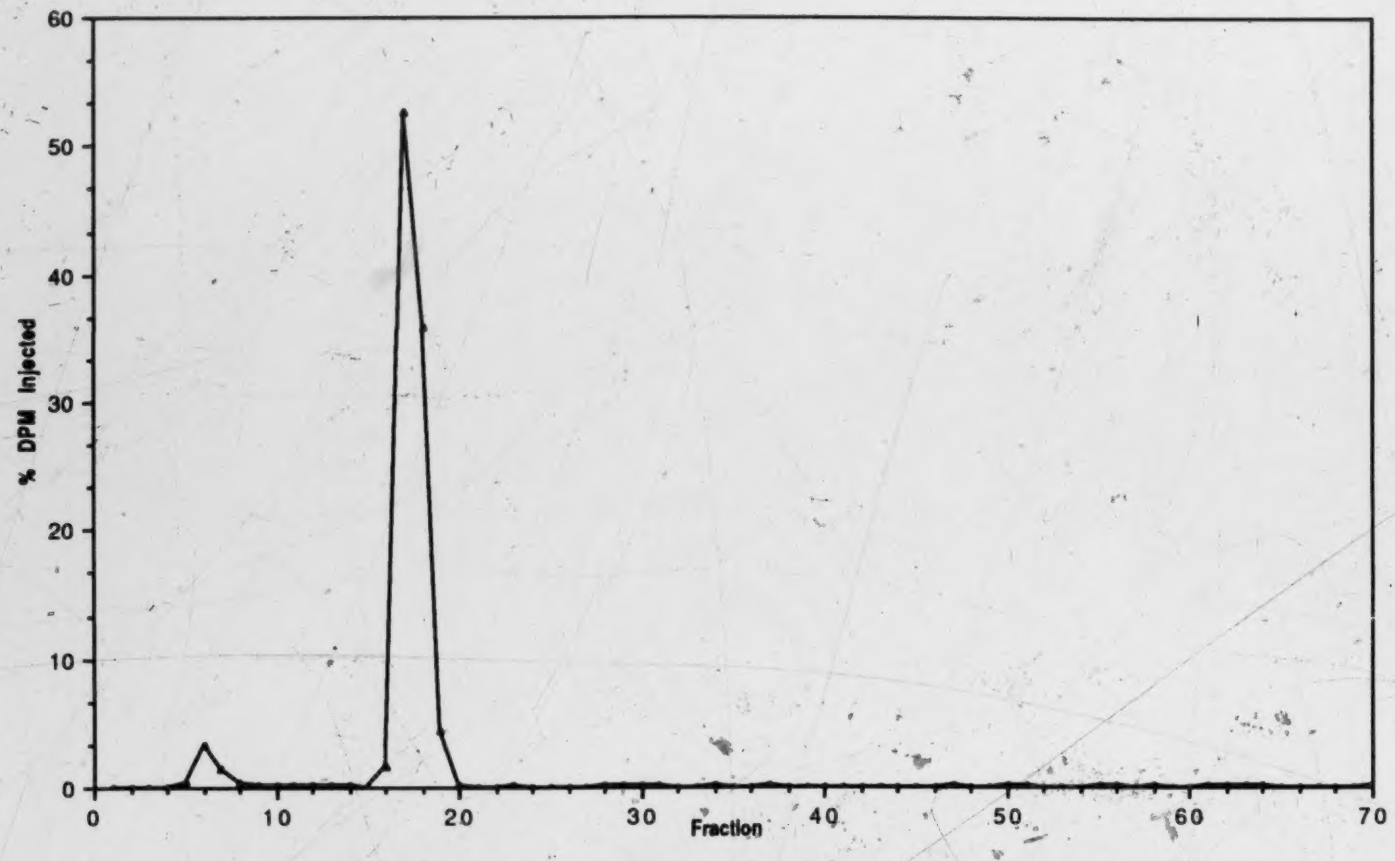

Figure 32. Analysis of Culture J Supernatant by HPLC and Fractionation: Solvent II
(Volume of each fraction = 0.35 mL; 0.35 mL = 0.35 min)
(Retention time of major peak (4-CP) = (Fraction) 17 x 0.35 min = 5.95 min)

3 x 30 µL injections. Total radioactivity recovery was 105%.

9.12.3.3. Analysis of culture J supernatant by HPLC and fractionation: Solvent III

Lastly, culture J was analyzed using solvent III. Again, the only significant peaks of radioactivity were that of acetate and 4-CP. Acetate in fraction 3 accounted for 4.5% of the radioactivity while 4-CP in fractions 19 to 22 accounted for 93% of the radioactivity. Again, Figure 33 shows the smaller acetate peak in comparison to 4-CP with a larger separation between the two compounds. Fractions were collected as 1.0 mL per minute up to 40 fractions. Total radioactivity applied was 4.7 x 10^3 DPM as one 60 µL injection. Total radioactivity recovery was 98%.

9.13. ^{14}C-4-Chlorophenol-containing cultures monitored through $^{14}CH_4$ production

Because of the rapid degradation of 4-CP in the previous set of radioactive 4-CP-containing cultures (section 9.9.), four additional cultures were prepared. This was to determine the stoichiometry of 4-CP degradation since these cultures were to be completely mineralized.

Each of the four cultures contained the following: 5 mL inoculum (sludge acclimated to 4-CP), 3.96 mL growth medium, 0.04 mL $Na_2S \cdot 9H_2O$, and 1 mL of 4-CP substrate to a final concentration of 30 mg/L. All four of the cultures, labelled M, N, P, and Q, had substrates analyzed at day 0. The intention was to leave three of the cultures untouched until the end of the experiment. Culture Q was monitored daily for substrate concentration, and every 2 to 3 days

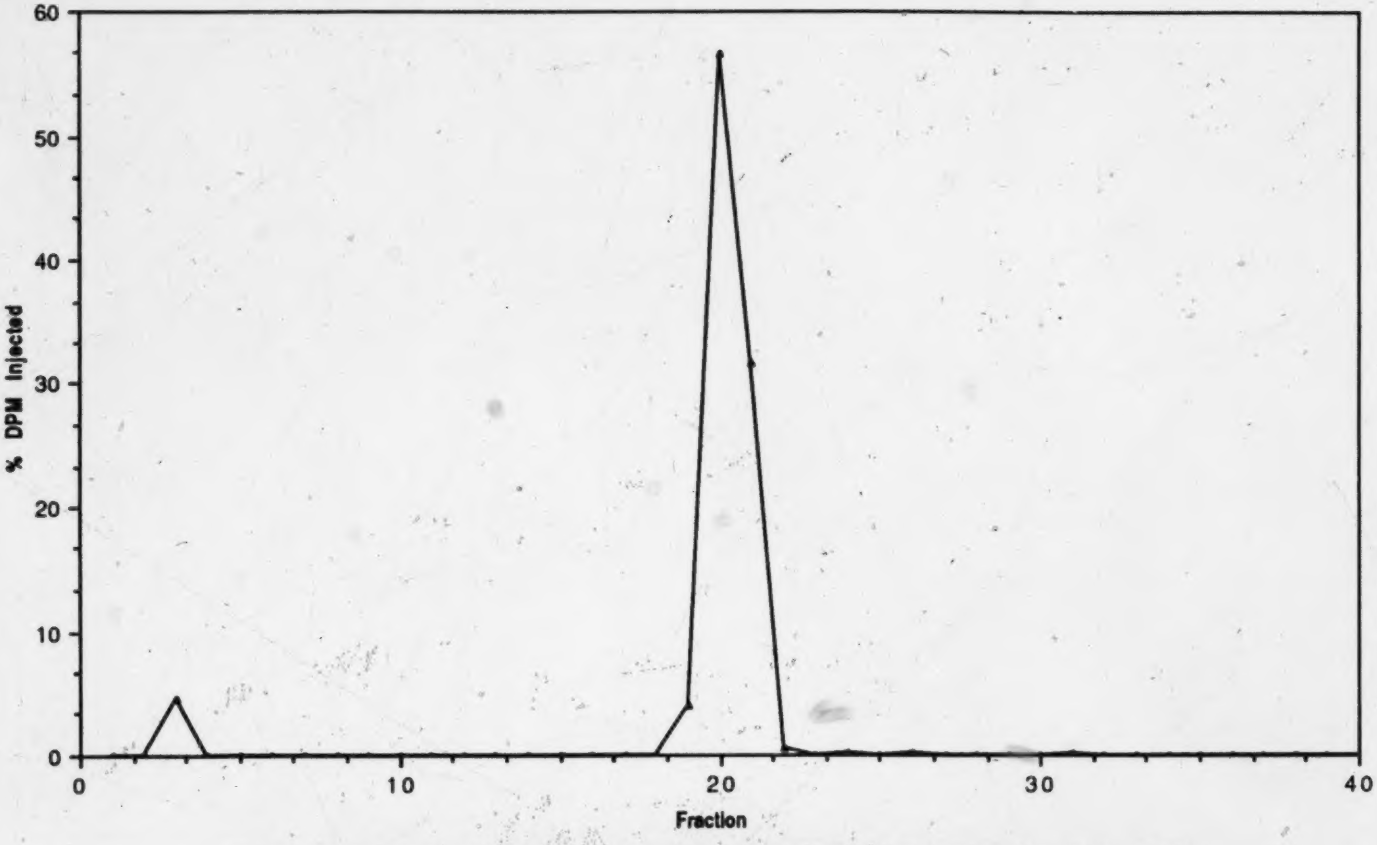

Figure 33. Analysis of Culture J Supernatant by HPLC and Fractionation: Solvent III
(Volume of each fraction = 1 mL; 1 mL = 1 min)
(Retention time of major peak (4-CP) = (Fraction) 20 x 1 min = 20 min)

for radioactive carbon dioxide and methane. This was done to determine the point at which the methane plateaued. The method of determining the methane plateau was based on work carried out by Roberts et al. (1987). The Duncans multiple range test was carried out to determine whether the methane had plateaued (Appendix G). After this point, the other three cultures were left to incubate for at least one week to ensure complete degradation of the substrate. As can be seen in Figure 34, culture Q degraded the 4-CP steadily at a rate of 0.3 mg/L/day, although a period of nearly 3 months was necessary to completely degrade the substrate.

Because methane was not produced in culture Q, the culture chosen to monitor, only the carbon dioxide was monitored. As can be seen (Table 12a), even after 133 days incubation, radioactive methane was still not produced. However, the other cultures were left for an additional two week period just in case the other three cultures had produced methane. After the radioactive methane and carbon dioxide in the headspace were determined, only two of the three remaining cultures (cultures N and P) had produced nearly the same amount of methane and carbon dioxide, although the $\%^{14}CH_4/\%$ total $^{14}CO_2$ ratios were still quite different (Table 12b). Culture M, on the other hand, had approximately the same amount of carbon dioxide as culture Q. As there was apparently a problem with the inoculum, total recovery of the radioactivity was not pursued. In addition, the cultures were all degrading at different rates because they did not have 0 mg/L of substrate at the same time even though the inoculum was from the same initial batch culture.

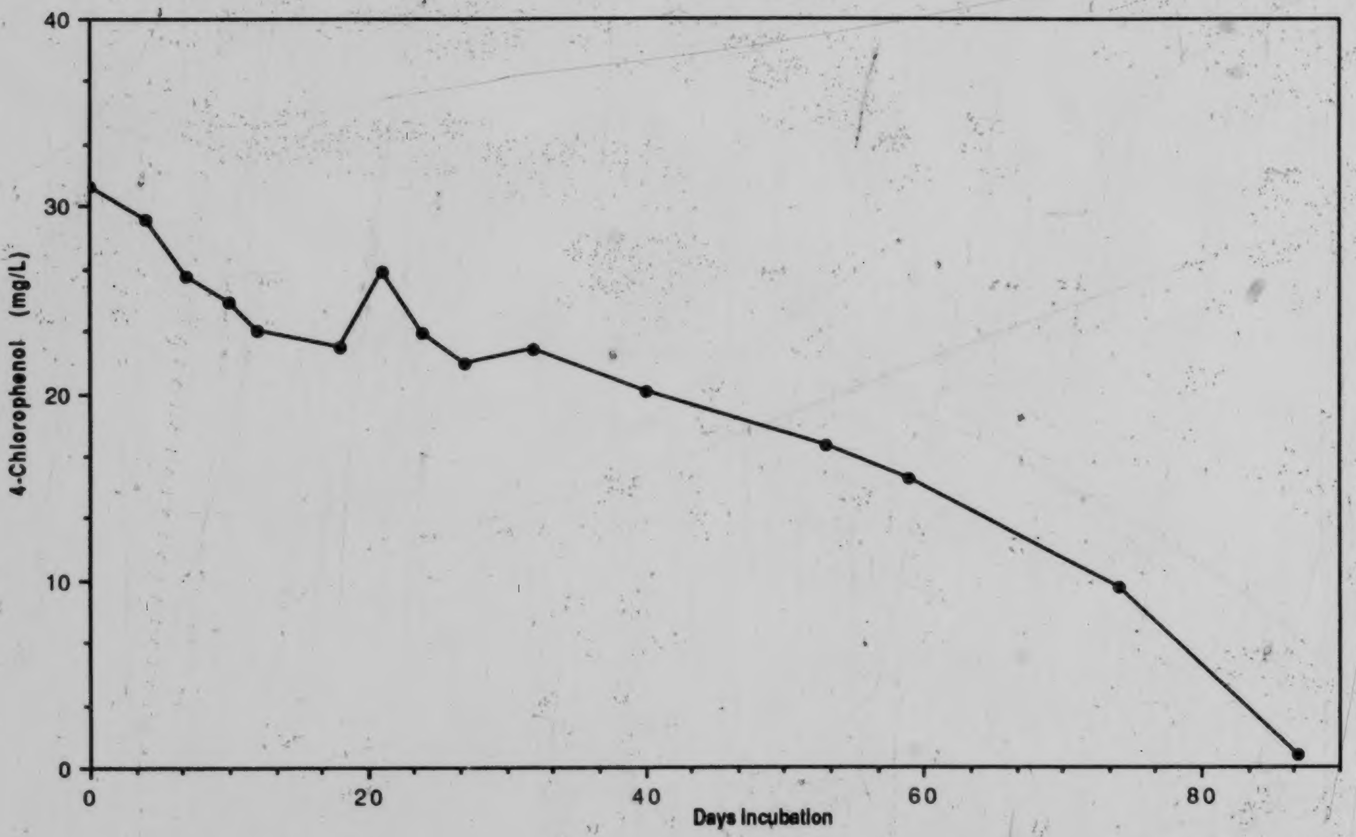

Figure 34. Degradation of 4-Chlorophenol by Culture Q

Table 12a. Determination of Methane Plateau for 4-Chlorophenol-Containing Culture Q

Days incubation	$\%^{14}CO_2 \pm 1SD$
126[a]	5.42 ± 0.30
130[b]	5.11 ± 0.46
133[c]	4.09 ± 0.57

[a] GPC Efficiency was 100%

[b] GPC Efficiency was 94%

[c] GPC Efficiency was 88%

Table 12b. Headspace Analysis of 4-Chlorophenol-Containing Cultures M, N, and P After 152 Days Incubation*

Culture	Initial 4-CP (mg/L)	$\%^{14}CO_2$	$\%^{14}CH_4\%$	$^{14}CH_4/\%^{14}CO_2$
M	33.4	4.60	none	-
N	33.7	13.3	25.4	1.91
P	32.9	11.1	31.0	2.79

* GPC Efficiency was 89%

9.14. Radioactive phenol-degrading cultures

Four phenol-degrading cultures were prepared as those described in section 9.13. In this case, the final substrate concentration was 22 mg/L and each contained 1 x 10^6 DPM of radioactive phenol. The cultures were labelled 1, 2, 3, and 4, and culture 4 was monitored for substrate concentration. As seen in Figure 35, a period of approximately two weeks was needed to degrade the phenol.

Culture 4 did not produce radioactive methane, and the culture was followed for a week to determine whether methane plateaued. The culture did appear to plateau based on observed methane values (Table 13a). However, a Duncans multiple range test was conducted to determine whether a plateau had actually been reached (Appendix G). Thus, according to this test, the percent radioactive methane in the headspace for days 15, 21, and 22 was the same within a 5% confidence interval, and days 21, 22, and 39 were the same within a 5% confidence interval. The percent carbon dioxide in the headspace for all days was equivalent by this test. Therefore, it was concluded that by day 39, methane had plateaued.

The total radioactivity recovery of these cultures displayed similar amounts of radioactive methane and carbon dioxide produced in the headspace (Table 13b) after 40 days incubation. The total recovery was better than previously, although approximately 20% of the radioactivity was still present in the solids and liquids.

This same experiment was carried out again using radioactive phenol and the same protocol. The only change for this set of

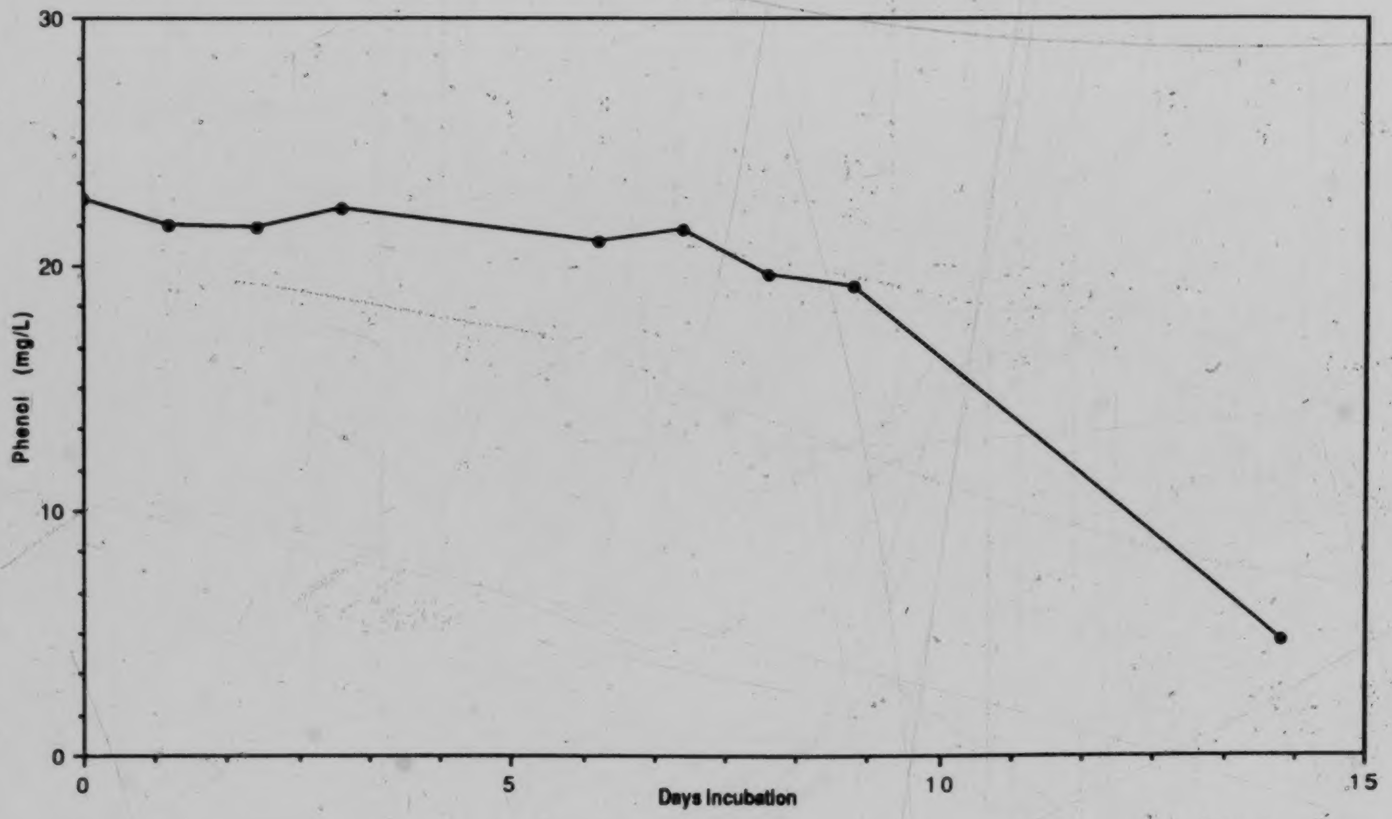

Figure 35. Degradation of Phenol by Culture 4

Table 13a. Determination of Methane Plateau for Phenol-Containing Culture 4

Days incubation	% $^{14}CH_4$ ± 1SD	% $^{14}CO_2$ ± 1SD
15[a]	22.6 ± 2.5	18.8 ± 1.8
21[b]	26.9 ± 4.6	17.1 ± 0.15
22[c]	29.0 ± 0.46	15.5 ± 3.1
39[d]	24.4 ± 1.8	13.6 ± 3.8

[a] GPC Efficiency was 97%
[b] GPC Efficiency was 98%
[c] GPC Efficiency was 88%
[d] GPC Efficiency was 88%

Table 13b. Total Recovery of Radioactivity From Phenol-Containing Cultures 1 to 4*

		Culture 1	Culture 2	Culture 3	Culture 4
Initial Phenol (mg/L)		25.3	24.4	24.6	22.7
% $^{14}CO_2$	Gas	15.4	18.6	15.5	26.3
	Liquid	31.6	28.1	31.1	26.3
	Total	47.0	46.7	46.7	40.8
% $^{14}CH_4$		26.2	31.5	27.9	25.2
% CH_4/% Total CO_2		0.56	0.67	0.60	0.62
% ^{14}C in Liquid		8.82	9.16	10.1	11.1
% ^{14}C in Solids		7.23	7.69	8.18	7.63
% Total Recovery†		89.3	95.1	92.7	84.7

* GPC Efficiency was 100%

† Total Recovery = % Total $^{14}CO_2$ + % $^{14}CH_4$ + % ^{14}C in Liquid + % ^{14}C in Solids

cultures was that the inoculum was from an established phenol-degrading digestor. This was done to ensure better degradation of the phenol so that less radioactivity would stay with the solids and liquids. These cultures were labelled 5, 6, 7, and 8. Culture 8 was followed to determine the methane plateau. The methane appeared to plateau in a week (Table 14a) as determined by the Duncans multiple range test (Appendix G).

These cultures were analyzed for the distribution of radioactivity after 13 days incubation (Table 14b). This set of cultures had nearly the same amount of total radioactive carbon dioxide and methane produced as cultures 1 to 4, even though the former set was incubated 27 days longer than the latter. However, the radioactivity in the solids and liquids was slightly higher in cultures 5 to 8. Again, there was considerable variation in the $\%^{14}CH_4/\%$ total $^{14}CO_2$ ratios both within and between each set of cultures.

9.15. Analysis of phenol-degrading culture 5 by HPLC and fractionation: Solvent III

Because so much of the radioactivity was present in the solids and liquids, the supernatant of one of the cultures was analyzed to determine whether radioactive phenol was still present.

Culture 5 had 22% of the radioactivity distributed between the solids and liquids. This culture was arbitrarily chosen from the second set of phenol cultures. The second set was chosen because these cultures had a higher percentage of radioactivity in the solids and liquids. Also, the radioactive stock solution added to the

Table 14a. Determination of Methane Plateau for Phenol-Containing Culture 8

Days incubation	%$^{14}CH_4$ ± 1SD	%$^{14}CO_2$ ±1SD
6[a]	26.3 ± 4.0	20.3 ± 0.88
10[b]	24.4 ± 3.9	19.6 ± 4.7
12[c]	26.1 ± 6.2	19.9 ± 3.7

[a] GPC Efficiency was 91%
[b] GPC Efficiency was 97%
[c] GPC Efficiency was 98%

Table 14b. Total Recovery of Radioactivity From Phenol-Containing Cultures 5 to 8*

		Culture 5	Culture 6	Culture 7	Culture 8
Initial Phenol (mg/L)		21.8	21.4	21.5	22.3
% $^{14}CO_2$	Gas	24.8	23.7	22.8	19.8
	Liquid	22.5	23.1	21.3	26.0
	Total	47.3	46.8	44.1	46.1
% $^{14}CH_4$		30.2	30.8	29.9	25.2
% CH_4/% Total CO_2		0.64	0.66	0.68	0.55
% ^{14}C in Liquid		11.0	10.8	10.6	10.8
%^{14}C in Solids		11.0	11.3	12.5	12.3
% Total Recovery†		99.5	99.7	97.1	94.4

* GPC Efficiency was 88%

† Total Recovery = % Total $^{14}CO_2$ + % $^{14}CH_4$ + % ^{14}C in Liquid + %^{14}C in Solids

cultures was the same one used for the initial set of phenol cultures labelled 1 to 4.

Solvent III was used for this analysis. As seen in Figure 36, the majority of the radioactivity was within fractions 3 and 4 accounting for nearly 40% of the radioactivity applied. The resultant peak had the same retention time as acetate. The total radioactivity applied to the column was 1.3 x 10^3 DPM as 1 x 160 µL injection. Fractions were collected as 1 mL per minute up to 40 fractions. Total radioactivity recovery was 80%. Culture 6 was also analyzed by the same method with generally the same results.

9.16. Analysis of 2-year-old radioactive phenol stock solution and radioactive 4-chlorophenol stock solution by HPLC and determination of volatility

The standards used in the recovery experiments were analyzed to determine whether any peaks other than phenol or 4-CP were detectable by HPLC. Also, the volatility of these compounds was analyzed during the flushing procedure. The aim was to determine whether any of the radioactivity from the standards could be trapped in Carbosorb after acidification if the standard had not been completely degraded.

Nine 58 mL serum bottles were prepared and contained the following: 9 mL growth medium, 0.1 mL $Na_2S{\cdot}9H_2O$, 0.9 mL substrate (one set with phenol and one set with 4-CP). One set of six had ^{14}C-phenol added to each bottle for a total radioactivity of approximately 9 x 10^5 DPM in each. The other set of three had ^{14}C-4-CP added to each for a total of 2.7 x 10^6 DPM in each bottle. On day 0, 3 x 10 µL samples were removed from each bottle and counted to

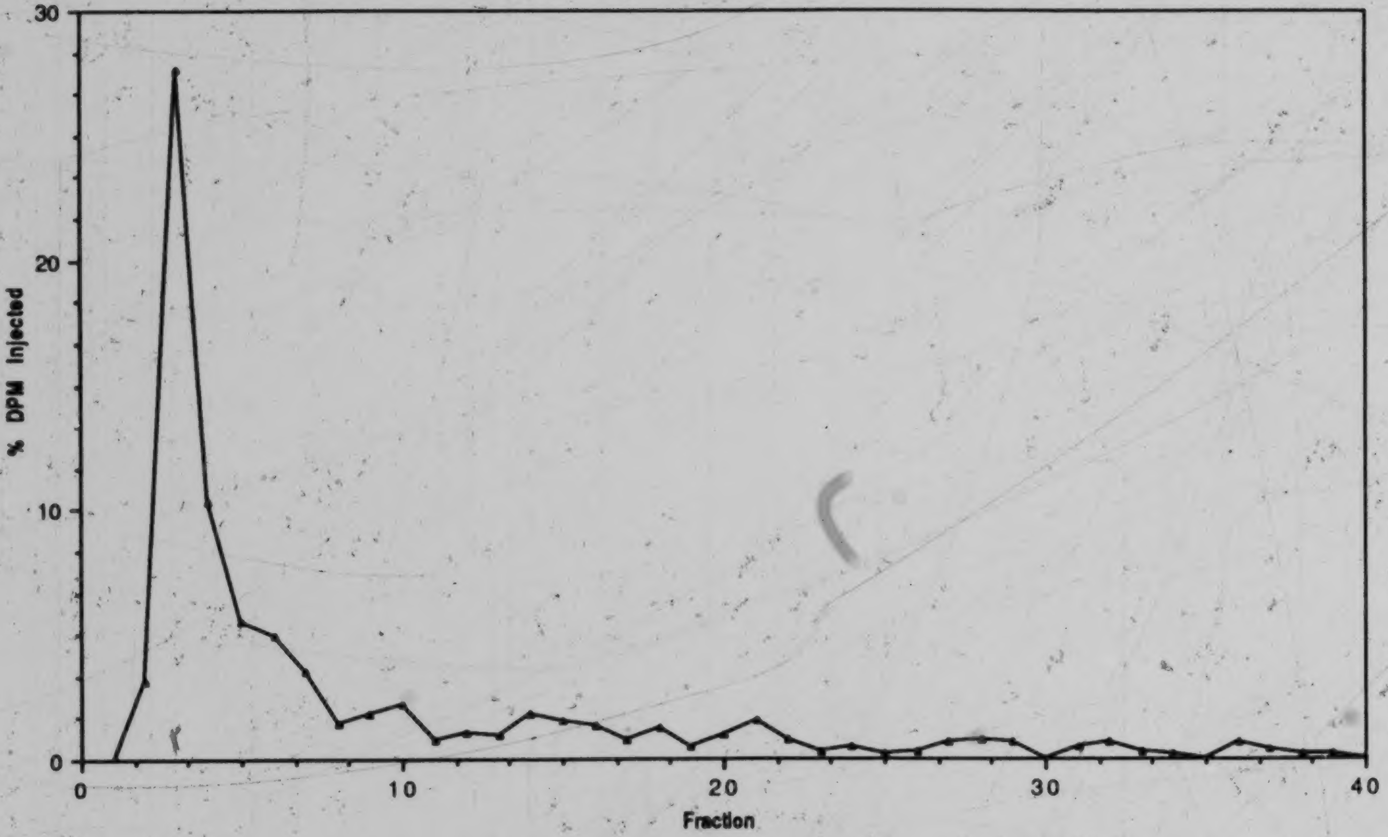

Figure 36. Analysis of Culture 5 Supernatant by HPLC and Fractionation: Solvent III
(Volume of each fraction = 1 mL; 1 mL = 1 min)
(Retention time of major peak (phenol) = (Fraction) 3 x 1 min = 3 min)

determine the initial radioactivity. Then, each of the six bottles was acidified and flushed for 3 x 10 minutes in the same manner as described in section 8.2.4.1. of Methods and Materials. The same experiment was repeated the following day. Samples were removed from each of the three bottles and analyzed on HPLC to determine whether peaks other than that of the substrate were present.

9.16.1. Radioactive phenol stock solution

9.16.1.1. Analysis of radioactive acidified phenol stock solution by HPLC and fractionation

The radioactive phenol stock solution was analyzed due to the high amounts of carbon dioxide recovered from the cultures. This was done to be certain that there was not some degradation of the phenol since it had been stored for 2 years. Phenol was analyzed using solvent III. This was to determine whether the 2-year-old standard had been microbially transformed to products that liberated carbon dioxide when acidified.

The stock solution was acidified to release any radioactive carbon dioxide present. A 20 µL sample of the stock solution was mixed with 20 µL of 6.2 M HCl in the fumehood. This solution was then applied to the column using solvent III.

3% of the radioactivity was located in fractions 4 to 7 while 24% was located in fractions 11 to 13. The plot (Figure 37) exhibited two peaks believed to be acetate (3%) and phenol (24%).

Fractions were collected as 0.5 mL per 0.5 minute up to 40 fractions. Total radioactivity applied was 4.3 x 10^3 DPM as 2 x 10 µL injections. Total radioactivity recovery was only 28%.

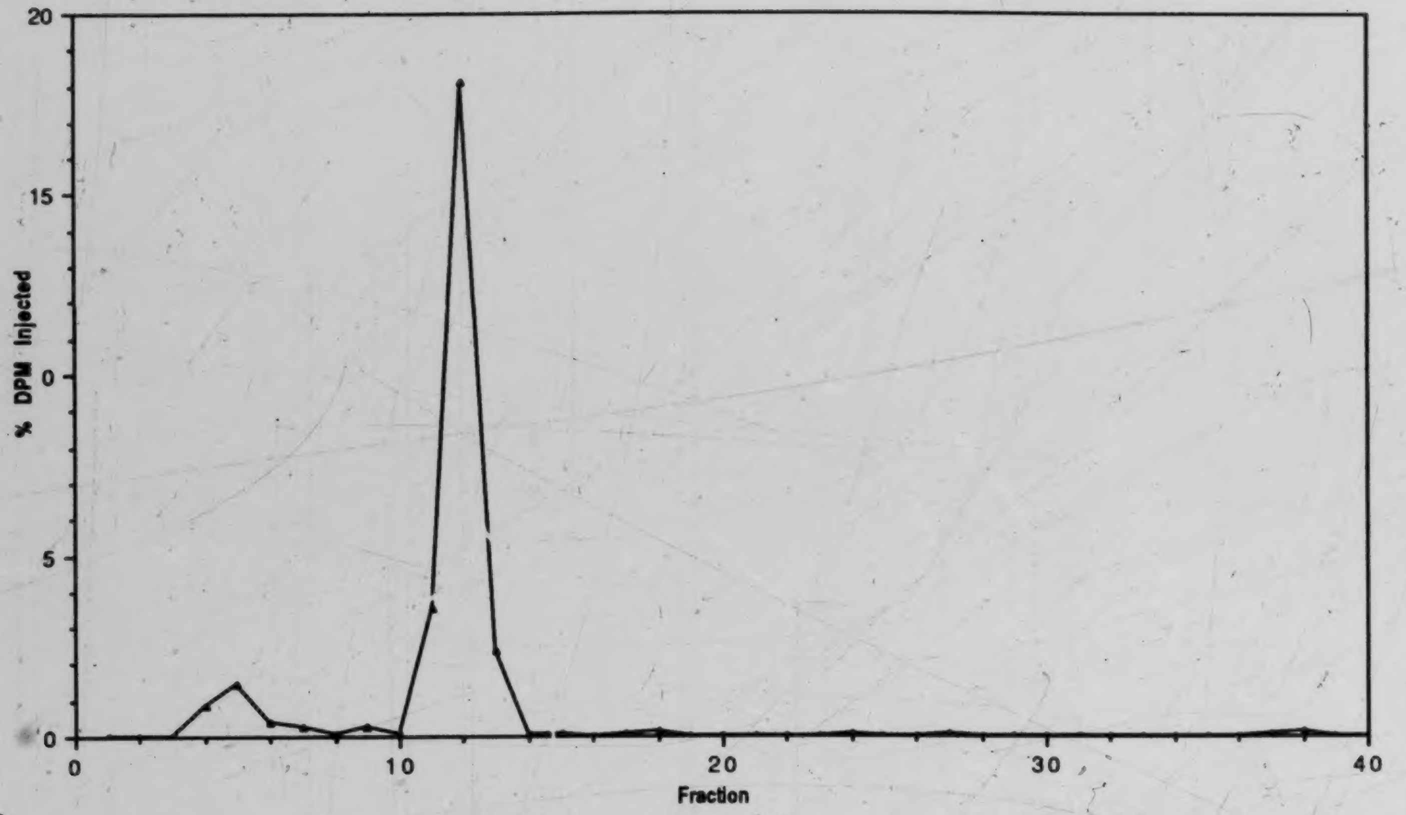

Figure 37. Analysis of Acidified Phenol Stock Solution by HPLC and Fractionation: Solvent III
(Volume of each fraction = 1 mL; 1 mL = 1 min)
(Retention time of major peak (phenol) = (Fraction) 12 x 1 min = 12 min)

9.16.1.2. Analysis of radioactive non-acidified phenol stock solution by HPLC and fractionation

This sample was taken from the stock solution and directly applied to the column. As seen, the highest amounts of radioactivity were located in fractions 5 to 9, accounting for 71% of the radioactivity. Also, there was a small peak of radioactivity before the phenol peak in fractions 2 to 4 that contained 8.0% of the radioactivity (Figure 38). This smaller peak had the same retention time as acetate. For triplicate analyses, fractions 5 to 9 each contained 70.7% of the added radioactivity.

Fractions were collected as 1 mL per minute up to 20 fractions. Total radioactivity applied was 1.8 x 10^3 DPM as 2 x 10 µL injections. Total radioactivity recovery was 81%.

9.16.2. Analysis of radioactive non-acidified 4-chlorophenol stock solution by HPLC and fractionation

One of the three 4-CP samples was taken directly from the stock solution and applied to the column. The highest amount of radioactivity was located in fractions 19 to 23 and contained 95% of the added radioactivity. For the chromatogram in Figure 39, fractions were collected as 1 mL per minute up to 35 fractions. The total radioactivity applied was 5.4 x 10^3 DPM as 2 x 10 µL injections with a total recovery of 97%. For triplicate analyses, fractions 19 to 23 contained 95.7% ± 0.53% of the added radioactivity.

9.16.3. Volatility of radioactive phenol and 4-CP stock solutions

Table 15 shows the results of flushing the six bottles. 8% of

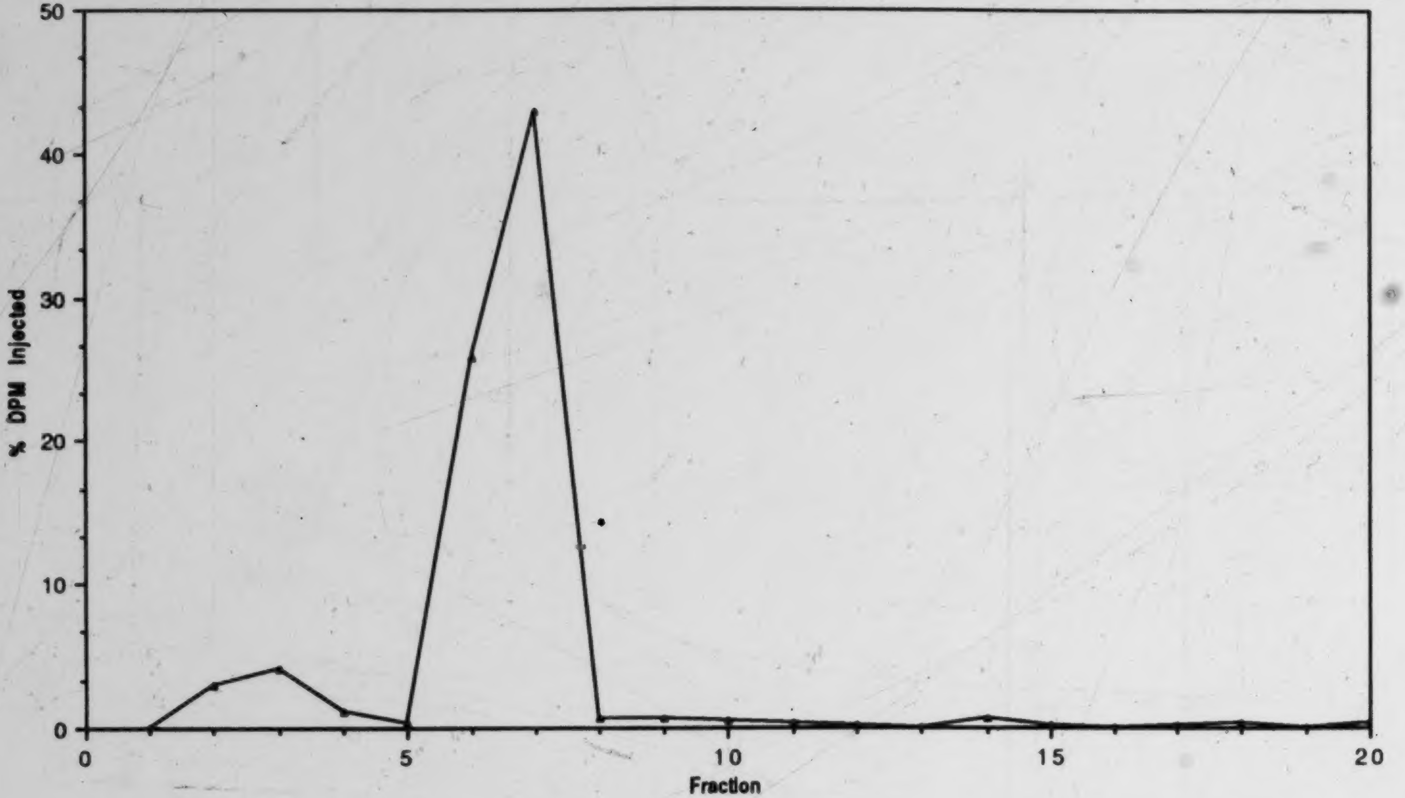

Figure 38. Analysis of Non-acidified Phenol Stock Solution by HPLC and Fractionation: Solvent III
(Volume of each fraction = 1 mL; 1 mL = 1 min)
(Retention time of major peak (phenol) = (Fraction) 7 x 1 min = 7 min)

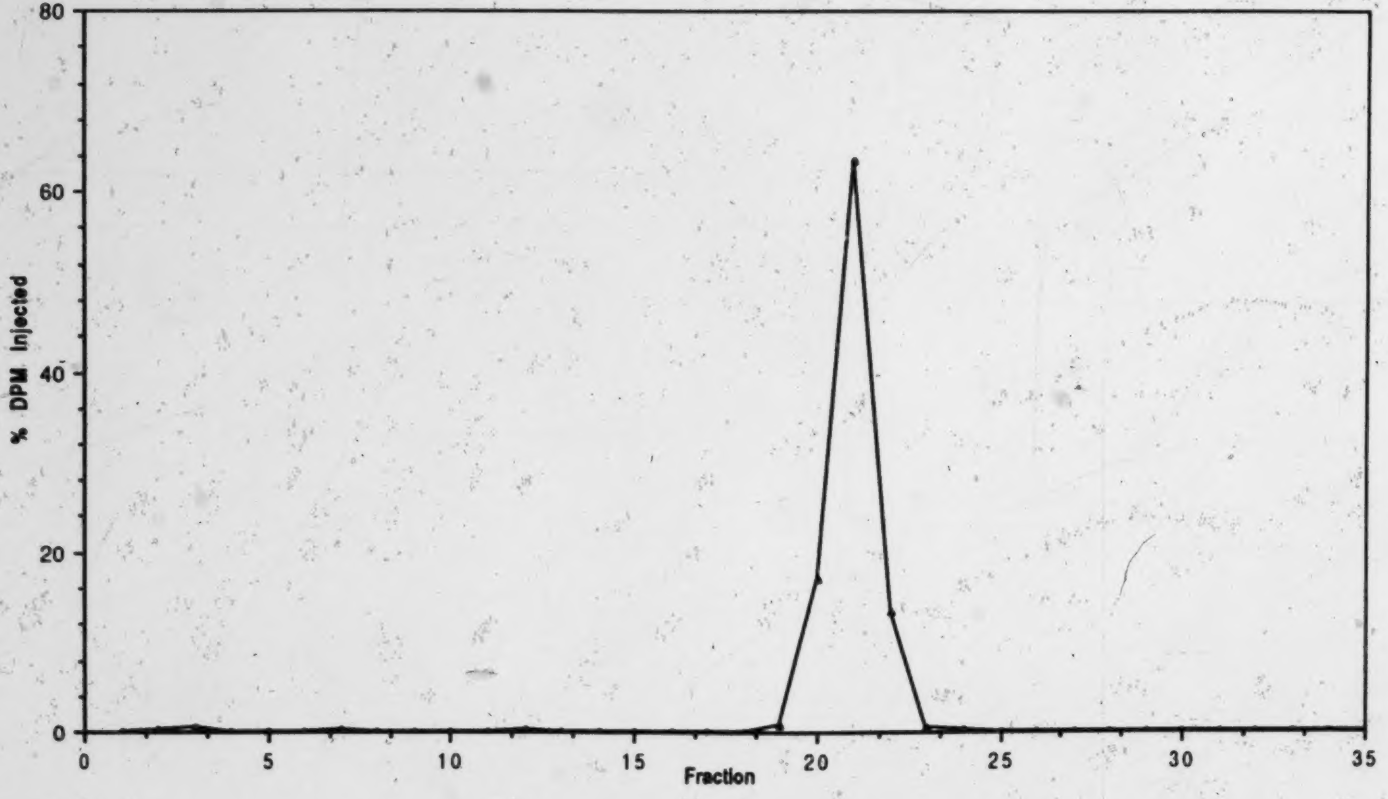

Figure 39. Analysis of Non-acidified 4-CP Stock Solution by HPLC and Fractionation: Solvent III
(Volume of each fraction = 1 mL; 1 mL = 1 min)
(Retention time of major peak (4-CP) = (Fraction) 21 x 1 min = 21 min)

Table 15. Volatility of the ^{14}C-Phenol and ^{14}C-4-Chlorophenol Acidified Stock Solutions

	Average % ^{14}C ± 1 SD	
	Phenol	4-CP
Flush 1	8.02 ± 1.71	0.07 ± 0.01
Flush 2	0.10 ± 0.02	0.01 ± 0.006
Flush 3	0.07 ± 0.01	0.01 ± 0.006

the phenol standard was trapped in Carbosorb. However, there was a negligible amount of 4-CP that was trapped in Carbosorb. From both the HPLC analysis and the flushing results, the phenol used was actually 71% pure after approximately two years storage at 4°C. From the HPLC analysis of 4-CP and the flushing results, 4-CP was still 96% pure after one year storage at 4°C.

9.17. Radioactive phenol-degrading cultures: Two concentrations

A second set of four radioactive phenol-degrading cultures was prepared using a new stock solution of ^{14}C-phenol. The phenol was not analyzed by HPLC because it had recently been obtained. This phenol was stored in diethyl ether which was dried down using a low stream of nitrogen gas over the surface of the ether.

These cultures were labelled 9, 10, 11, and 12 with culture 12 used to monitor the methane plateau. The inoculum for this experiment was another phenol-degrading digestor, but not the same one used for cultures 5 to 8. After 11 days incubation, the headspace of culture 12 was analyzed. However, it was not possible to detect methane or carbon dioxide in the headspace. The headspace was again analyzed on day 14 and again on day 18. As can be seen from Table 16a, the methane and carbon dioxide production was very low. Therefore, it was decided to leave the remaining cultures for approximately one week and then analyze them.

The total radioactivity recovery from this set of cultures (Table 16b) averaged 74.4% ± 3.4%. However, more than half of the radioactivity remained in the solids and liquids.

Table 16a. Determination of Methane Plateau for Phenol-Containing Culture 12

Days incubation	%$^{14}CH_4$ ± 1SD	%$^{14}CO_2$ ± 1SD
11	ND	ND
14[a]	7.03 ± 2.8	8.88 ± 4.7
18[b]	5.87 ± 1.5	5.92 ± 0.75

ND = Not detected

[a]GPC Efficiency was 89%

[b]GPC Efficiency was 99%

Table 16b. Total Recovery of Radioactivity From Phenol-Containing Cultures 9 to 12*

		Culture 9	Culture 10	Culture 11	Culture 12
Initial Phenol (mg/L)		23.6	23.0	23.0	22.7
% $^{14}CO_2$	Gas	ND	2.81	4.26	ND
	Liquid	-	5.59	5.04	-
	Total	7.70	8.40	9.30	8.63
% $^{14}CH_4$		2.74	3.47	4.55	1.97
% CH_4/% Total CO_2		0.36	0.41	0.49	0.23
% ^{14}C in Liquid		35.3	34.9	37.2	35.8
%^{14}C in Solids		23.7	29.0	24.1	30.7
% Total Recovery†		69.4	75.8	75.2	77.1

ND=Not detected

- = Not calculated

* GPC Efficiency was 93%

† Total Recovery = % Total $^{14}CO_2$ + % $^{14}CH_4$ + % ^{14}C in Liquid + %^{14}C in Solids

One more experiment was conducted using the same batch of radioactive phenol used for cultures 9 to 12. Two sets of five 38 mL serum bottles were prepared at two phenol concentrations. One concentration was 14.1 mg/L and the other was 28.2 mg/L. Each bottle contained the following: 9 mL inoculum, 0.9 mL phenol, and 0.1 mL $Na_2S \cdot 9H_2O$. The inoculum used was sludge from the same phenol-degrading digestor used for cultures 5 to 8. In this case, one of the five cultures was used to monitor the substrate while the other four were sacrificed as soon as the substrate was depleted.

The cultures with the lower concentrations were labelled A1, B1, C1, and D1, and the higher concentrations were labelled A2, B2, C2, and D2. The total radioactivity recovery of the lower concentrations was tabulated in Table 17. These cultures were sacrificed after one week incubation. Again, a considerable amount of the radioactivity stayed with the solids and liquids although recovery was better than 80%. Table 18 shows the recovery results for the higher phenol concentrations. These cultures were left to incubate for 52 days. This did not seem to help because approximately 60% of the radioactivity was in the liquids and solids. The extra value of solids + liquids in Table 18 was another analysis of the recovery technique. Three 0.1 mL samples were removed from each acidified culture and counted to determine whether there was any loss in the liquids and solids analyses, which there was not.

A summary of all radioactivity recoveries for both ^{14}C-4-CP-containing cultures and ^{14}C-phenol-containing cultures are shown in Tables 19 and 20, respectively. The determination of theoretical percentages of methane and carbon dioxide in the headspace is

Table 17. Total Recovery of Radioactivity From Phenol-Containing Cultures A1 to D1*

Culture		A1	B1	C1	D1
Initial Phenol (mg/L)		11.1	11.2	13.2	13.7
% $^{14}CO_2$	Gas	3.89	1.75	1.41	1.43
	Liquid	10.7	4.92	4.90	4.43
	Total	14.6	6.66	6.31	5.86
% $^{14}CH_4$		5.54	3.55	3.36	4.16
% CH_4/% Total CO_2		0.38	0.53	0.53	0.71
% ^{14}C in Liquid		38.1	43.0	42.2	42.3
%^{14}C in Solids		24.5	30.9	30.1	29.9
% Total Recovery†		82.7	84.1	82.0	82.2

* GPC Efficiency was 95%

† Total Recovery = % Total $^{14}CO_2$ + % $^{14}CH_4$ + % ^{14}C in Liquid + %^{14}C in Solids

Table 18. Total Recovery of Radioactivity From Phenol-Containing Cultures A2 to D2*

Culture		A2	B2	C2	D2
Initial Phenol (mg/L)		31.2	28.3	31.2	30.1
% $^{14}CO_2$	Gas	2.64	1.49	1.40	1.74
	Liquid	12.0	3.64	5.30	4.95
	Total	14.6	5.13	6.70	6.69
% $^{14}CH_4$		6.48	3.83	4.19	4.07
% CH_4/% Total CO_2		0.44	0.75	0.63	0.61
% ^{14}C in Liquid		29.8	32.1	33.0	34.8
% ^{14}C in Solids		23.4	35.0	36.1	32.3
% ^{14}C in Liquids + Solids		56.2	63.1	68.4	60.5
% Total Recovery†		74.3	76.1	80.0	77.9

* GPC Efficiency was 84%

† Total Recovery = % Total CO_2 + % $^{14}CH_4$ + % ^{14}C in Liquid + % ^{14}C in Solids

Table 19. Summary of Radioactivity Recoveries for ^{14}C-4-Chlorophenol-Containing Cultures

	Culture									
	C^1	D_a^1	D_b^1	K^2	G^2	H^2	J^2	M	N	P
%$^{14}CO_2$ gas	-	-	-	15.6	8.60	1.88	6.89	-	-	-
liquid	-	-	-	23.5	23.5	4.15	9.69	-	-	-
total	37.0	43.0	37.7	39.1	32.1	6.03	16.6	4.60	13.3	25.4
%$^{14}CH_4$	19.0	12.6	12.6	25.8	21.5	4.04	28.0	none	25.4	31.0
%CH_4/%Total CO_2	0.51	0.29	0.33	0.66	0.67	0.67	1.69	ND	1.91	1.22
% ^{14}C in liquid	1.90	ND	1.12	5.55	12.7	47.9	29.4	-	-	-
% ^{14}C in solids	0.31	ND	1.04	1.84	3.04	7.59	5.75	-	-	-
%Total recovery	58.2	55.6	52.5	72.3	69.3	65.6	79.8	-	-	-

^{1} = Preliminary radioactivity recovery procedure (Section 9.10.1.)

^{2} = Second radioactivity recovery procedure (Section 9.10.2.)

- = No analysis was done

ND = Not determined

Table 20. Summary of Radioactivity Recoveries for ^{14}C-Phenol-Containing Cultures

	Culture									
	1	2	3	4	5	6	7	8	9	10
%$^{14}CO_2$ gas	15.4	18.6	15.5	26.3	24.8	23.7	22.8	19.8	ND	2.81
liquid	31.6	28.1	31.1	26.3	22.5	23.1	21.3	26.0	-	5.59
total	47.0	46.7	46.7	40.8	47.3	46.8	44.1	46.1	7.70	8.40
%$^{14}CH_4$	26.2	31.5	27.9	25.2	30.2	30.8	29.9	25.2	2.74	3.47
%CH_4/%Total CO_2	0.56	0.67	0.60	0.62	0.64	0.66	0.68	0.55	0.36	0.41
% ^{14}C in liquid	8.82	9.16	10.1	11.1	11.0	10.8	10.6	10.8	35.3	34.9
% ^{14}C in solids	7.23	7.69	8.18	7.63	11.0	11.3	12.5	12.3	23.7	29.0
%Total recovery	89.3	95.1	92.7	84.7	99.5	99.7	97.1	94.4	69.4	75.8

	Culture									
	11	12	A1	B1	C1	D1	A2	B2	C2	D2
%$^{14}CO_2$ gas	4.26	ND	3.89	1.75	1.41	1.43	2.64	1.49	1.40	1.74
liquid	5.04	-	10.7	4.92	4.90	4.43	12.0	3.64	5.30	4.95
total	9.30	8.63	14.6	6.66	6.31	5.86	14.6	5.13	6.70	6.69
%$^{14}CH_4$	4.55	1.97	5.54	3.55	3.36	4.16	6.48	3.83	4.19	4.07
%CH_4/%Total CO_2	0.49	0.23	0.38	0.53	0.53	0.71	0.44	0.75	0.63	0.61
% ^{14}C in liquid	37.2	35.8	38.1	43.0	42.2	42.3	29.8	32.1	33.0	34.8
% ^{14}C in solids	24.1	30.7	24.5	30.9	30.1	29.9	23.4	35.0	36.1	32.3
%Total recovery	75.2	77.1	82.7	84.1	82.0	82.2	74.3	76.1	80.0	77.9

- = No analysis was done

ND = Not determined

shown in Appendix F.

9.18. Methane production from non-radioactive phenol

This experiment was conducted to determine whether there was a difference in the milliliters of methane produced per mg of phenol when a low concentration of 25 mg/L and a high concentration of 300 mg/L phenol were added to culture. The inoculum used was from a phenol-degrading digestor. Seven 58 mL serum bottles were prepared and contained the following: 3 mL inoculum, 5.9 mL growth medium, 0.1 mL $Na_2S \cdot 9H_2O$, and 1.0 mL freshly boiled, deoxygenated water or phenol to a final concentration of 300 mg/L. Another seven 158 mL serum bottles contained the following: 36 mL inoculum, 82 mL growth medium, 1.0 mL $Na_2S \cdot 9H_2O$, and 1.0 mL freshly boiled, deoxygenated water or phenol to a final concentration of 25 mg/L. Three of each set of bottles were controls, and the rest were the tests.

Transducer standards were also prepared. The cultures were set to atmospheric pressure, the substrate was added, and phenol concentrations were analyzed at time 0. The methane was analyzed every 2 days in the controls for each set and in only one of the test cultures of each set. When the methane plateaued in the test culture, methane production in the rest of the test cultures was analyzed and compared to predicted methane production. To determine predicted methane production, the following relationship was used: 1 mg of phenol produced 0.75 mL methane (Fedorak, 1984).

The calibration curve for phenol was linear. However, there were two curves for both the low and high concentrations of phenol

(Figure 40). Therefore, the curve corresponding to the lower phenol concentrations was used for cultures G1, H1, J1, and K1, and the curve corresponding to the higher phenol concentrations was used for cultures G2, H2, J2, and K2. The methane plateau for the low phenol concentrations was reached by day 14 of the incubation while the high concentrations reached their plateau by day 21 at which time the experiment was terminated (Figure 41). The methane was dry methane at standard temperature and pressure (STP).

Using the equivalent of 0.75 mL methane/mg phenol, all net methane production values were much higher than predicted, except for culture D1 (Table 21). Using the formula based on the equation by Buswell and Mueller (1952), 1 mg of phenol produced 0.83 mL methane. As can be seen, cultures G1, H1, J1, and K1 were below predicted values except for J1 which was only a difference of 0.2 mL. However, cultures G2, H2, J2, and K2 had approximately 20% more methane than predicted by the Buswell equation. The average mL methane produced per mg phenol was 0.81 mL ± 0.072 mL for cultures G1, H1, J1, and K1 which was very close to the expected 0.83 mL methane/mg phenol predicted. However, the average mL methane produced per mg phenol was 1.00 mg/mL ± 0.028 mg/mL for cultures G2, H2, J2, and K2. Using either the equivalents of 0.75 mL/mg or 0.83 mL/mg, cultures G2, H2, J2, and K2 produced much more methane than predicted. There was apparently a difference in methane production between the two phenol concentrations.

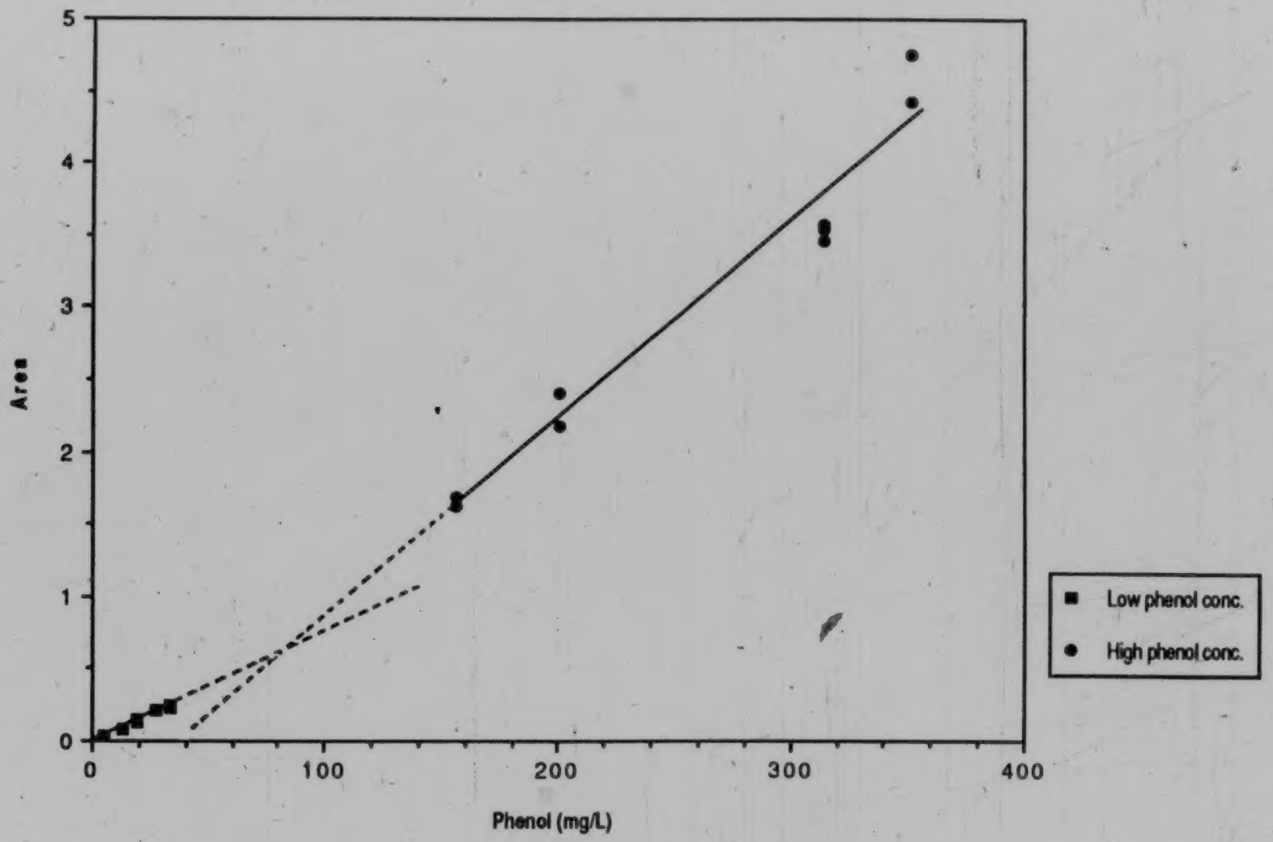

Figure 40. Calibration Curves for Low and High Phenol Concentrations

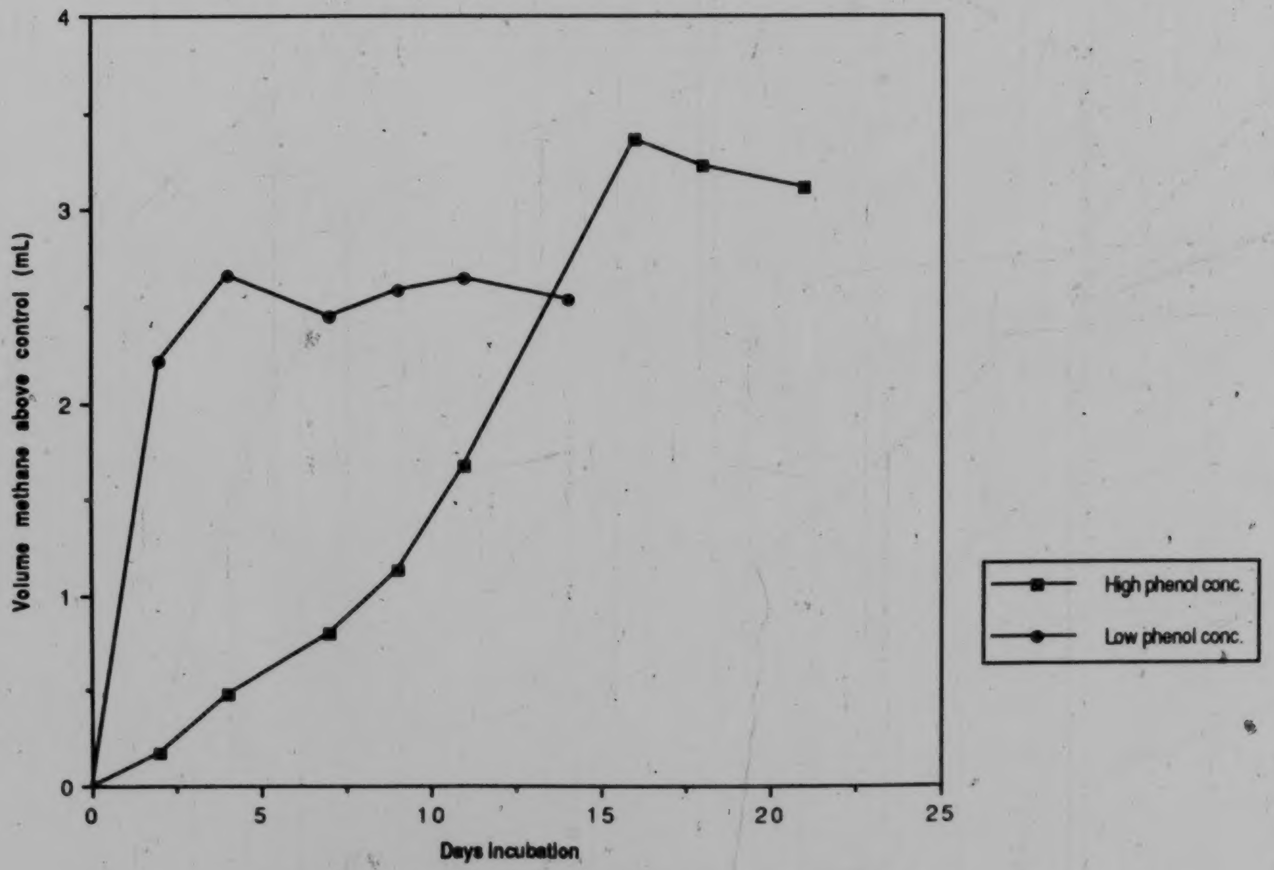

Figure 41. Methane Plateaus for High and Low Phenol Concentrations
(Dry methane at STP)

Table 21. Methane Production From Phenol-Containing Cultures*

Culture	Phenol conc. (mg/L)	mg Phenol Present	Predicted Methane Production (mL)		Observed Methane Production (mL)	Percent of Theoretical		Observed mL methane per mg phenol produced
G1	24.8	2.98	2.23[a]	2.47[b]	2.50	112[a]	99[b]	0.84
H1	28.4	3.41	2.56[a]	2.83[b]	2.67	104[a]	94[b]	0.78
J1	25.4	3.05	2.29[a]	2.53[b]	2.72	119[a]	108[b]	0.89
K1	29.2	3.50	2.63[a]	2.91[b]	2.54	97[a]	87[b]	0.73
G2	298	2.98	2.23[a]	2.47[b]	3.11	139[a]	126[b]	1.04
H2	309	3.09	2.32[a]	2.56[b]	3.06	132[a]	120[b]	0.99
J2	310	3.10	2.33[a]	2.57[b]	3.02	130[a]	118[b]	0.97
K2	313	3.13	2.35[a]	2.60[b]	3.11	132[a]	120[b]	0.99

[a] Using 0.75 mL methane per mg phenol (Fedorak, 1984)

[b] Using 0.83 mL methane per mg phenol (Buswell and Mueller, 1952)

* Dry methane at STP

10. Discussion

10.1. 4-Chlorophenol enrichment cultures

The August 4-CP enrichment cultures displayed difficulty in degrading the initial substrate added. It was thought that the 4-CP was recalcitrant. However, a period of 6 months was necessary to deplete the substrate to non-detectable levels. These enrichments contained a considerable amount of organics and presumably biomass in comparison to the later enrichments. The October and November enrichments contained, at best, 39% of the biomass present in the August enrichments (Table 5).

It was not expected that the 2,4-diCP enrichment cultures would take such a long period of time to transform the substrate, because it has been demonstrated that the presence of an ortho chlorine was more readily removed. That is, after a 6 month incubation period, the 2,4-diCP enrichment cultures had not completely removed the 4-CP formed as a result of dechlorination at the ortho position of 2,4-diCP.

The sludge used for all enrichment cultures was added within 24 hours after removing it from the treatment plant digestor. Some researchers (Shelton and Tiedje, 1984a; Boyd et al., 1983) used sludge stored at 4°C as their inoculum, which was then diluted to 10%. Boyd et al. (1983) added 5 mg/L 4-CP which was almost completely degraded in 4 months. Boyd and Shelton (1984), however, used fresh sludge with a concentration of 20 mg/L 4-CP, which was very close to the concentration used in the enrichments for this work. They found that 4-CP was degraded in nearly 4 weeks. The wide variability in results was not only because of techniques, but was also a reflection of the variability in the composition of sludges which directly influenced the bacterial populations present. For this research, the enrichments contained 96% sludge as the inoculum. Using the sludge immediately is optimal because the microbial population is most active.

The variability in sludges depends on the efficiency of the treatment plant also. That is, the sludge composition could be affected by factors such as the waste sources, the time of day, and even the season of the year (Buhler et al., 1973). An illustration of this variability can be seen by comparing the results of Hrudey et al. (1987a) with the results of this research. Hrudey et al. (1987a) found that 4-CP up to concentrations of 100 mg/L in 50% sludge was degraded in 16 to 20 weeks. However, in this research, the sludge was obtained from the same treatment plant, yet 25 mg/L of 4-CP was degraded in 6 months, and in some cases greater than one year. Also, the high level of organics and biomass in the August enrichments was also an important factor in the rapid biodegradation in comparison to the other enrichments. Even among batch cultures, there were differences because the batch cultures were not degrading at the same rate.

10.2. Adsorption of 4-chlorophenol and 2,4-dichlorophenol to sludge

10.2.1. Enrichment cultures containing radioactive substrates

The distribution of radioactivity in the radioactive enrichment cultures was surprising (Tables 6a and 6b). Such a large percentage of the radioactivity residing in the solids portion was not expected.

This may have been caused by adsorption to the suspended organic matter. However, this did not seem plausible because the non-radioactive 4-CP-containing enrichment cultures contained nearly 25 mg/L 4-CP in culture at time 0, as expected. If adsorption was occurring, it should also have occurred in the non-radioactive cultures containing the same source of sludge. That is, the non-radioactive 4-CP-containing cultures would have been expected to contain approximately 13 mg/L or 50% of the initial substrate added. Johnson and Young (1983) did find a decrease of 40% in the first few hours of incubation within 2,4-diCP-containing cultures. However, their initial concentration was four times the concentration used for this research.

Dobbs et al. (1989) used calculated adsorption coefficients (Kp') for toxic organic compounds to determine an equation correlating the Kp' to the K_{ow}. The calculated Kp' was based on adsorption to wastewater solids. Based on the equation derived from the correlations (Appendix D), the log Kp' for 2,4-diCP was 3.0, compared to the observed value of 2.2 for this work. Thus, according to this correlation, there was agreement between the observed and calculated values of log Kp' for 2,4-diCP. Applying the correlation to 4-CP, the log Kp' is 2.5 (using a K_{ow} of 2.4).

The high amount of radioactivity in the solids could have been because of the technique of analyzing the radioactivity. That is, the amount of radioactivity in the solids was determined by the difference in radioactivity between the suspension (solids plus supernatant) and the supernatant. The supernatant was analyzed by removing a very small sample and centrifuging. Samples of the liquid above the dense pellet were removed and counted. The solids plus supernatant analysis may have been too high because the samples removed from the culture contained a large amount of particulate matter because of the thick suspension.

10.2.2. Non-radioactive 2,4-dichlorophenol-containing cultures

The sludge adsorption studies using 2,4-diCP at a concentration of 25 mg/L were conducted to compare an active (non-sterile) and inactive (sterile) sludge used as a 50% inoculum. The sterile sludge was used to eliminate any biodegradation and to observe only adsorption to organics. In experiment I, the sterile water sample ranged from 16 mg/L to 18 mg/L, approximately 10 mg/L less than expected. It was suspected that this may have been caused by evaporation because there were no adsorption sites present as would be expected with a culture containing sludge. Applying the Henry's law constant for 2,4-diCP, only a negligible amount of 2,4-diCP should be found in the gas phase (Appendix E). The pH of the growth medium was 7.0 ± 0.1, whereas the pH of fresh sludge was 7.2 to 7.6 (Nowak, personal communication). Therefore, the pH of the culture would be between 6.9 and 7.6. The pKa of 2,4-diCP is 7.89 (Table 1); therefore, with the pKa very close to the pH of the culture, approximately 50% of the compound would be dissociated, and thus unable to adsorb to particulate matter.

An analysis of the stock solution in section 9.5.1.2. was done to make sure that there was no error in preparation of the stock solution. A secondary purpose was to determine the accuracy of removing µL samples with a Hamilton syringe. First, a sample of the stock solution removed without rinsing the syringe showed more deviation from the average than the second part of the experiment in which the syringe was rinsed several times with the stock solution to be analyzed. This was because in the first part, a small amount of rinse water would still be present in the needle of the syringe. Consequently, a 1 µL sample could be diluted slightly thus explaining the higher deviation in this part of the experiment. Thus, the second method proved to be the method of choice and was in fact the one used throughout this project. However, the concentration of 2,4-diCP was 2 to 3 mg/L less than expected. The average of 22.9 mg/L was taken to be the actual concentration. However, even with a concentration of 22.9 mg/L, the sterile water experiment produced slightly lower values than expected. It should be noted that the last six points of the sterile water curve were only taken with one of the sterile water bottles because one of the bottles was lost (Figure 7). This might explain the wide variation in this curve.

The sterile and non-sterile cultures in part I both had concentrations of 9 mg/L in solution after the 7 hour period. Thus, in both cases, approximately 14 mg/L of 2,4-diCP was adsorbed in a very short period of time. Initially, there was a 2 mg/L difference in concentration for both types of sludges, with the sterile cultures being slightly higher. Based on calculations by Woods (1984) in Appendix D, the expected loss from solution was 11.3 mg/L 2,4-diCP on the basis that the initial concentration of 2,4-diCP added to the cultures was 15.7 mg/L, the concentration of the sterile water sample. It was assumed that there may have been a problem either with analysis of the initial concentration or addition of the 2,4-diCP to the cultures.

Centrifugation of the samples in part I would have affected the observed concentrations if the solubility of 2,4-diCP was affected. In water, the solubility of 2,4-diCP is 4500 mg/L at 25°C (Table 1). If some of the 2,4-diCP was precipitating, it would have concentrated to the bottom of the centrifuge tube. However, because variability in replicates ranged from 1 to 5 mg/L, this difference was too small to conclude that one of the sludges adsorbed to a greater degree than another. Thus, the two sludges adsorbed 2,4-diCP to the same degree.

The cultures in part II of this experiment proved to be more erratic in terms of the results (Figure 8). Again, the sterile water sample initially contained approximately 17 mg/L, which was much lower than expected. In this case, the first and last four points of the sterile water samples were taken as single points. In this instance, the water samples ranged from 19 mg/L to 11 mg/L. Experimentally, it would have been more favorable for both parts of this experiment if duplicate or even triplicate water samples were taken at exactly the same sampling period.

An interpretation of the sludge cultures can be made, however, in terms of the trends seen. Both the sterile and non-sterile cultures had an initial 2,4-diCP concentration of approximately 19 mg/L which was still 4 mg/L lower than expected. The non-sterile cultures decreased by 6 mg/L in a 5 hour period, whereas the sterile cultures decreased by 4 mg/L in the same amount of time. However, after a 24 hour period, the non-sterile cultures had increased to nearly the same level observed after the 5 hour period. The rapid increase in the 2,4-diCP cultures suggested a biological mechanism or an adsorption-desorption mechanism that may be biologically facilitated that is not present in the sterile cultures. Also, problems with sampling or analysis may have resulted in the erratic numbers observed. Since these cultures were taken directly from the bottle without allowing the cultures to settle, clogging of the syringe with the sludge particles would make it difficult to remove the supernatant. Also, leaking at the septum of the gas chromatograph would result in less liquid entering the column, with the actual concentration appearing less than it should be.

Johnson and Young (1983) also tested 2,4-diCP at a concentration of 100 mg/L with sterile and non-sterile sludge. They found 40% and 31% removal for the non-sterile and sterile cultures, respectively. However, they did not specify their method of sterilizing their cultures. Part II of the adsorption experiment for this project had 43% and 35% removal for the non-sterile and sterile sludges, respectively. These values were very close to those found by Johnson and Young (1983). In part I, 65% and 52% removal were found for the non-sterile and sterile cultures, respectively. However, the inherent problem in these experiments was the loss of 2,4-diCP from the sterile water controls which did not contain any sludge or particulates that could have adsorbed the 2,4-diCP.

Extrapolation of these results to 4-CP must be done cautiously, because the 2,4-diCP molecule has an extra ortho chlorine in relation to the 4-CP molecule. This extra chlorine enhances the adsorptive properties of 2,4-diCP in comparison to 4-CP. This is evident in the log K_{ow} values of 2.44 and 3.23 for 4-CP and 2,4-diCP, respectively. The higher K_{ow} of 2,4-diCP translates to a greater affinity for organics. Thus, there would be some adsorption of 4-CP to organics, but not to the same degree as that found for 2,4-diCP. This was shown when comparing the log Kp' of 2,4-diCP which was 3.0 to the log Kp' of 4-CP which was 2.5 based on Dobbs et al. (1989) as shown in Appendix D. Thus, according to calculated values of log Kp', 2,4-diCP would adsorb to a greater degree than 4-CP.

10.3. Dilution experiments containing 2-chlorophenol or 4-chlorophenol

The dilution experiments of the 2-CP-containing and 4-CP-containing cultures were carried out prior to the preparation of the August enrichment cultures which was the reason the 4-CP-containing cultures were incubated for only 34 days. If the 4-CP-containing cultures had been left longer, an effect of the dilution would have been noted. It would probably have been the same as that seen for 2-CP, because this substrate is much easier to degrade than 4-CP, and dilutions of the 2-CP-containing cultures did not enhance degradation. The lack of methane production in the 4-CP-containing cultures also lead to the conclusion that no degradation was occurring.

The 2-CP-containing cultures were used primarily as a reference, because degradation was known to be more rapid than 4-CP, and intermediates had been detected in earlier research. The dilutions of 2-CP-containing cultures had a pronounced effect on 2-CP degradation, particularly the 1/8 dilution (Figure 11). In comparison to the undiluted cultures, the time for complete degradation in the 1/8 dilution increased by nearly 17 days. The 1/4 dilution increased its lag time by one week, whereas the 1/2 dilution increased its lag time by only 3 days. Thus, there were components within the inoculum that were important for degradation to proceed quickly. If there were inhibitory compounds or metabolites present, there were not enough to impede 2-CP degradation. If the 4-CP-containing cultures had been left to incubate longer, degradation might have taken longer than 6 months just for the undiluted cultures, assuming that there were no components that inhibit 4-CP degradation. An important factor to consider when interpreting the dilution experiments is the bacterial populations present in the sludge. Not only are compounds necessary for growth being diluted, but the active bacterial populations are also being diluted. Thus, this variable in conjunction with growth components will have a negative effect on degradation. Also, the inoculum for this experiment contained less TSS than that for the October enrichments, suggesting that even the undiluted culture of 4-CP could have taken more than one year to degrade this substrate.

In addition to the effect on degradation, the 2-CP-containing cultures also displayed phenol production, but production took longer with increasing dilutions (Figure 12). The phenol production peaked when the 2-CP concentration was below 3 mg/L for all dilutions, suggesting inhibition of phenol degradation with increased concentrations of 2-CP. That is, nearly all of the 2-CP was dechlorinated prior to phenol degradation. Thus, a higher 2-CP to inoculum ratio as seen with the most dilute cultures (1/4 and 1/8) may have had an inhibitory effect on dechlorination and subsequent phenol production.

The average peak concentration of phenol produced in the dilutions reached 13.5 mg/L, which was 4.8 mg/L lower than expected assuming a one to one molar conversion of 2-CP to phenol. The discrepancy between theoretical (Appendix B) and observed values was because the initial concentration of 2-CP was not analyzed. Therefore, the actual concentration may have been slightly lower. Also, because the phenols in the cultures were not analyzed on a daily basis, the phenol peak may have been higher for some of the cultures and thus was missed on a day that substrates were not analyzed. Another factor to consider is that the phenol was being degraded during the experiment, and some may have been degraded before all of the 2-CP was dechlorinated.

The methane production showed that there was some biological activity present (Figure 13). Methane production was caused by the formation of phenol as a result of 2-CP dechlorination. Ideally, cultures used as controls containing no substrate should have been used to show background methane production caused by the organics in the inoculum. Hrudey et al. (1987b) found no difference in methane production between the controls and the 2,4-diCP cultures. If it was assumed that the 2-CP was dechlorinated to phenol which then yielded methane, a molar ratio of 1 to 3.5 (2-CP to methane) would result. Thus, with an initial concentration of 25 mg/L, only 0.7 mL of methane would be produced (Appendix C). The slight increase seen in the curves of Figure 13 was probably this methane. Also, the points at which slight increases in methane production occurred in Figure

13, did not correspond to the decreases in phenol concentration in Figure 12. For example, in Figure 12, the phenol concentration began to decrease for the undiluted culture on day 7. In the corresponding Figure 13, the increase in methane production occurred on approximately day 21.

The 2-CP-fed digestor degraded 25 mg/L of this substrate within 10 days (Figure 9), exhibiting an active bacterial population. This experiment showed a dechlorination rate of 2.88 mg/L/day which converts to 0.14 mg/day. This value was lower than the 0.29 mg/day obtained by Hrudey et al. (1987a) for a 2-CP-degrading culture containing 97 mg/L 2-CP initially. This shows the variability from one culture to another. The digestor appeared to be performing well. However, shortly after this experiment, the digestor was no longer degrading 2-CP. This was indicative of the sensitivity of bacterial cultures, most probably the dechlorinating-microorganisms, because this digestor had been operating for approximately two years.

10.4. Dechlorination of 2-chlorophenol

After the previous experiment, the dechlorination of 2-CP was further studied. The two phenol-fed digestors were chosen as sources of inocula because they had well-established bacterial populations capable of degrading phenol. Phenol digestor 3 had been maintained much longer than Phenol digestor 4. Phenol digestor 3 had been maintained for approximately 2 years, whereas Phenol digestor 4 had been maintained for less than 6 months. Surprisingly, the fresh sludge dechlorinated the 2-CP completely at a rate of 0.22

mg/day (Figure 10). The others did not dechlorinate as quickly. It was at this point that it was decided the 2-CP-fed digestor had become inactive. The Phenol 3 digestor did however have more than 100 mg/L phenol present initially. Initially, this culture dechlorinated faster than the fresh sludge but then levelled off. The lag times in both phenol digestor cultures were probably because of the fact that the digestors had a large number of phenol-degraders present because of the maintenance on phenol. Dechlorinating-microorganisms that were present were likely in much lower numbers and not active because the digestors were maintained on a non-chlorinated substrate. The fresh sludge, however, was obtained directly from a treatment plant digestor meaning that a variety of substrates were present including chlorinated compounds, which would mean a more active population of dechlorinators than found in phenol digestors.

10.5. 4-Chlorophenol degradation

The first time any proof of biodegradation was noted during this project was with the cultures containing radioactive 4-CP or 2,4-diCP. Although the evidence was only qualitative, it confirmed the work of previous researchers. Boyd and Shelton (1984) found that >90% of the added radioactivity was converted to $^{14}CO_2$ and $^{14}CH_4$ using radiolabelled 2-CP, 4-CP, and 2,4-diCP. Mikesell and Boyd (1986) found that 66% of ^{14}C-pentaCP was mineralized to 27.4% $^{14}CO_2$ and 38.3% $^{14}CH_4$. In the former case, both $^{14}CO_2$ and $^{14}CH_4$ were observed, however, in the latter case, the $^{14}CH_4$ was calculated based on the observed $^{14}CO_2$.

The first radioactive 2,4-diCP sample analyzed by HPLC had only 13.2% of the radioactivity recovered. A major loss of radioactivity was caused by the acidity of the solvent used. A pH of 3 would result in the loss of carbon dioxide to the atmosphere. At the time of analysis, the cultures had incubated for 6 months. Therefore, any intermediates formed should have subsequently been mineralized.

The enrichment culture used as the inoculum for the study of the 4-CP degradation rate was chosen randomly from the twenty August enrichment cultures. Culture F degraded 40 mg/L 4-CP in approximately 3 weeks. The degradation rate of 1.8 mg/L/day was the same as the rate of 1.8 mg/L/day for the second addition of 4-CP (Figure 20). This would be expected with an active culture especially if the second substrate addition was added very close to the time at which the first addition was depleted. If there had been a delay in the second addition, there might have been a lower rate with the second addition of 4-CP because it is not clear how quickly the chlorophenol-degraders would need to have the substrate added in order to maintain the same level of activity.

Cultures G, H, and J were prepared to observe 4-CP degradation rates as well as intermediates. All three cultures displayed a steady substrate degradation. Initially, degradation rates were similar among the cultures. However, after the addition of the second amount of 4-CP plus the radioactive 4-CP, there was a significant increase in the degradation rate. Cultures G and J had a rate increase of 2.1 mg/L/day and 2.3 mg/L/day (Figures 21 and 23), respectively. However, culture H appeared to decrease much faster than the other two cultures (Figure 22). This was, again, a reflection of the variability within a group of cultures from a single inoculum source. Although the inoculum was from the same batch culture, the inability to obtain a homogeneous population for each culture resulted in varying degradation rates.

10.6. Recovery of radioactivity from ^{14}C-4-chlorophenol-containing cultures

Total recovery of the radioactivity from cultures was important primarily to determine the stoichiometry involved in 4-CP degradation. Also, an account of the total radioactivity was necessary to determine the distribution of radioactivity within the cultures after or during degradation, with the assumption that it was possible to account for 100% radioactivity.

The preliminary procedure used for total radioactivity recovery resulted in less than 60% recovery (Table 9). A major problem with this procedure was the size of the culture that was flushed. The 50 mL culture, upon the addition of HCl, produced copious amounts of gas which were flushed into two vials which each contained 9 mL of fluor and 1 mL of Carbosorb. This resulted in some of the fluor entering the tubing connecting the scintillation vials. This was where some of the carbon dioxide was lost because the carbon dioxide would then adhere to the Carbosorb in the lines as well as the Carbosorb in the vials. This could result in considerable losses, depending on the amounts of carbon dioxide produced. Also, if there was not enough Carbosorb present in the scintillation vials, the adsorption capacity of the Carbosorb may have been exceeded.

The large proportion of radioactivity found in the liquids and solids was not expected, however, because this batch culture had incubated for 6 months. That is, it was assumed that the substrate in these cultures had been depleted as seen in the cultures that had non-radioactive 4-CP.

The second procedure was an alteration of the preliminary procedure. The second procedure was carried out to determine whether a better radioactivity recovery overall, and of the carbon dioxide in particular, could be obtained. There was no improvement in recovery of radioactivity. That is, even with duplicates from the same culture, a total radioactivity recovery of less than 60% was again noted (Table 9). A portion of the radioactivity in both of these procedures was present in the solids and liquids. The concentration of solids was too high for the scintillation counter. As a result, the counting efficiency was reduced. Although not as pronounced, the same problem was noted for the supernatant. That is, even a 600 µL sample was too opaque because of the brownish color of the supernatant.

The third procedure was an alteration of the second procedure to improve recovery of radioactivity from solids and liquids. The main difference was in the procedure used for the solids. The vacuum filtration was to remove excess radioactive liquid that would obscure the actual amount of radioactivity in the solids. However, by washing the filter with water, excess liquid was removed as well as any radioactivity that may have been adsorbed to the sludge particles. Cultures K, G, H, and J had a much higher radioactivity recovery than previous attempts, but approximately

20% to 30% of the radioactivity was unaccounted for (Table 10). The loss was from the washes of the solids which were not collected.

To prove that the loss of radioactivity was in the washes, the frozen culture H was analyzed. The procedure was again changed to enable collection of the washes. It was difficult to collect the washes with a vacuum filtration apparatus, therefore, a Swinex filter containing a 0.45 µm pore diameter filter was used. As suspected, the loss of radioactivity appeared to be within the washes (Table 11). Washing the filters, however, may shift the distribution slightly. That is, it may appear that more radioactivity is present in the supernatant rather than in the solids. Although, for the purposes of this research, the concern was to determine how much of the radioactivity remained in the solids plus the liquids in comparison to the amount of substrate that was actually mineralized.

The third radioactivity recovery procedure was found to be the best one in terms of recovering most of the added radioactivity (Table 20). Removal of portions of the headspace to be analyzed by the GPC was not a problem. The possible losses at this stage were through leakage through the stopper of the serum bottle and an improperly tightened septum at the injection port of the GC attached to the GPC. Also, leakage of the sample may have occurred if the tubing between the GC and GPC and within the GPC was not examined for leaks prior to analyzing the sample. One area that may have caused problems was with the removal of the sample from the acidified culture that was to be filtered through a 0.45 µm pore diameter filter. That is, the cultures needed to be diluted before filtering otherwise the filter would become plugged. A portion of the acidified culture was removed and diluted in distilled water. A 1 mL portion of the diluted culture was then filtered. Therefore, losses may have occurred when transferring the sample to distilled water because a homogeneous sample was not easy to remove from a culture containing many particulates. After washing the filter into the scintillation vials, the Swinex filter was taken apart, and there was some liquid inside the filter even though air was pushed through to remove as much of the liquid as possible. Some losses may have occurred here, although these would have been minimal, because the last wash of the filter did not contain a large amount of radioactivity.

A further analysis of the final procedure used to re-analyze culture H was on the solids interference, if any (section 9.11. of Results). Even with a small sample of solids to be counted in the LSC, there was a 10% loss in counting efficiency. The difference of 1.83% was not deemed significant, and, therefore, this final procedure was not altered any further.

The radioactive phenol and 4-CP standards were acidified and flushed to determine whether any losses in radioactivity recovery could occur from volatilization of these compounds. The phenol used was the 2-year-old phenol stock solution. About 8% of the radioactivity was trapped in the Carbosorb (Table 15). This large amount was expected because HPLC chromatograms showed the presence of a radioactive material with the same retention time as acetate. This could obscure results from cultures that have not completely degraded phenol, because a portion of the radioactivity from acetate would be counted as part of the radioactive carbon dioxide during the flushing procedure. The cultures for these experiments primarily contained radioactive acetate. Fedorak et al. (1982) found that 5.2% ^{14}C-labelled acetate was carried into the scintillation vials containing Carbosorb. However, the 5.2% of the radioactivity found in the cultures would not be a significant loss of radioactivity. Approximately 0.1% of the 4-CP volatilized after acidification (Table 15), which was negligible, and, therefore, would not have caused any obscurity in radioactivity recovery results obtained from cultures that still contained radioactive 4-CP in solution.

10.7. Intermediates of 4-chlorophenol degradation

The radioactive culture 4-CP A was analyzed three times using solvent I to determine the radioactive profile of the supernatant. That is, any intermediates that appeared would be present extracellularly. The first and second analyses recovered 72% (Figure 14) and 102% (Figure 15) of the applied radioactivity, respectively. Yet, in both cases, 60% of the radioactivity was recovered as radioactive 4-CP as confirmed by applying authentic 4-CP to the HPLC column. The third analysis was for 30 minutes rather than 5 minutes to determine whether any radioactive peaks came off the column after 5 minutes, and to improve recovery of radioactivity. However, the longer analysis time did not show any additional peaks, or any improved radioactivity recovery (Figure 18). The lower radioactivity recovery was probably because of the low amount of 306 DPM applied to the column, whereas previously, twice as much was applied. The amount of 306 DPM also made the chromatogram appear to have extra peaks. Thus, a peak reading only 3 DPM above background appeared as a peak containing 1% of the total radioactivity applied. Ideally, the more radioactivity applied, the better. The first analysis with both the 4-CP A plus standard 4-CP sample again showed only the 4-CP peak with no difference in peak characteristics (Figure 17).

Cultures G, H, and J were the primary focus regarding the presence of any intermediates because these cultures were stopped while actively degrading 4-CP. Also, these cultures had a large amount of radioactivity in the supernatant, some of which was presumed to be intermediates (Table 10). Although there was still some radioactive 4-CP present in the supernatant of 4-CP A, it was not actively degrading 4-CP because the 4-CP was still present after 6 months incubation.

Culture G was first analyzed using solvent I (Figure 24). Surprisingly, a large amount of the radioactivity appeared to be 4-CP. However, there appeared to be a small peak just prior to 4-CP. It was at this point that solvent II was formulated. The organics in solvent II were increased to increase the retention times of phenol and 4-CP. The second analysis of culture G improved the profile by separating 4-CP from the initial peak (Figure 25). Because it was known that acetate elutes quickly, radioactive acetate was applied to determine whether this initial peak had the same retention time as authentic acetate. The problem with the second solvent was that if radioactive phenol was eluting, the phenol peak would appear in fraction 10. If there was some phenol present, it could have been obscured by the tail of the acetate peak. Therefore, solvent III was used to resolve the acetate, phenol, and 4-CP so that there was no interference between peaks. When culture G was analyzed using solvent III, the major peaks noted were that of 4-CP and presumably acetate (Figure 27). If phenol was present, it was in such a low concentration that it was not possible to detect it. Authentic acetate was analyzed using solvent III again to determine whether the initial peak had the same retention time as authentic acetate, which it did (Figure 28). Confirmation of the 4-CP peaks was done using non-radioactive 4-CP. Culture J resulted in the appearance of the same peaks seen for culture H (Figures 32 and 33).

When culture H was analyzed using solvent II, the same peaks of acetate and 4-CP appeared (Figure 29). However, some fractions that were assumed to be only background were reading approximately 0.11% which corresponded to 126 DPM, which was quite high. This may also have been radioactivity that had remained on the column from previous analyses because the radioactivity recovery was greater than 100%. Culture H was analyzed with solvent III to determine whether phenol was present (Figure 30). It was thus concluded that phenol was not present in culture H.

The three cultures degraded the 4-CP to different levels and this was exhibited to some degree through the amounts of radioactive acetate and 4-CP collected during HPLC analysis. That is, 74%, 32%, and 38% of the 4-CP in cultures G, H, and J were degraded, respectively. HPLC analysis with solvents II and III displayed amounts of 4-CP increasing in the following order: H > J > G. Therefore, culture G had the least amount of radioactive

4-CP because it had degraded the most 4-CP. HPLC analysis with solvents II and III showed the reverse for the amounts of acetate produced. This would be expected because more degradation should lead to more intermediate production.

The absence of any detectable phenol in both radioactive and non-radioactive 4-CP-containing cultures made it difficult to elucidate the biodegradation pathway. The presence of a compound presumed to be radioactive acetate, in addition to the presence of radioactive carbon dioxide and methane, proved that biodegradation was occurring. However, because acetate is a two carbon compound, this proved that the ring was cleaved at some point during degradation. The area of interest in this project was the manner in which the chlorine was removed during degradation. Thus far, dechlorination at the ortho positions of 2-CP, 2,3-diCP, 2,4-diCP, and 2,6-diCP had been documented with the resultant production of phenol, 3-CP, 4-CP, and phenol, respectively (Hrudey et al., 1987b).

The only instance in which phenol was detected as a degradation product of 4-CP under anaerobic conditions was in research conducted by Gibson and Suflita (1986). However, this was seen in cultures containing inoculum from pond sediment and a methanogenic aquifer. When sewage sludge was used, 4-CP was not degraded after 3 months. This illustrated the diversity of microbial populations in different inocula.

Mikesell and Boyd (1986) studied pentaCP degradation and proposed a biodegradation pathway. However, even though phenol was not identified as an intermediate, their proposed pathway showed phenol as a transitional product that was consumed as rapidly as it was formed, and was thus non-detectable.

In this project, phenol was not positively identified, and therefore was not conclusively stated to be an intermediate during 4-CP degradation. However, even though ortho chlorines have been shown to be more easily dechlorinated than para chlorines, it would still be more energetically favorable to displace a chlorine with a hydrogen group from a water molecule rather than cleave the aromatic ring prior to dechlorination. Displacement of a chlorine with a hydrogen has been demonstrated during dechlorination of 3-CB to benzoate (Schink, 1986). The formation of phenol from 4-CP would be more plausible because phenol has been shown in this research to be more rapidly degraded than 4-CP under anaerobic conditions. Thus, it would be more favorable to convert the 4-CP to a product that is more conducive to degradation.

Under anaerobic conditions, the aromatic ring can be oxidized by the addition of oxygen via oxygenases. Vogel and Grbic-Galic (1986) found that toluene and benzene underwent an anaerobic oxidation in which the source of oxygen was water. Although this area has not been extensively studied, it could be a route in the biodegradation of chlorophenols, possibly by displacement of the halogen to form catechols.

A recovery of greater than 100% of total radioactivity applied to the HPLC column was a reflection of the column characteristics and, thus, a drawback with the HPLC analysis. That is, when other samples were analyzed for a certain period of time, it was possible that a small amount of radioactivity may have been retained on the column because its retention time was longer than the period of analysis. Therefore, when another sample was applied later, this radioactivity would elute with the next sample, obscuring radioactivity recovery. Measurement error with the syringe may also have been a factor, however, the former explanation was more likely the reason for the recoveries greater than 100%.

In all cases of HPLC analysis, the chromatograms displayed many extra peaks in addition to the known radioactive peaks. These extra peaks were based on UV detection. However, these peaks did not contain significant amounts of radioactivity to warrant further investigation. These were possibly compounds present in the original inoculum or breakdown products as a result of bacterial degradation of other organics present in the inoculum.

10.8. Stoichiometry of ^{14}C-4-chlorophenol-containing cultures and ^{14}C-phenol-containing cultures

10.8.1. ^{14}C-Phenol-containing cultures

Before attempting to determine the distribution of radioactivity in the the 4-CP-containing cultures that were being monitored through a methane plateau, cultures containing radioactive phenol were prepared and the radioactivity was recovered. These results were compared with the stoichiometry of phenol degradation that had been previously studied and documented (Healy and Young, 1978; Healy and Young, 1979; Neufeld et al., 1980; Knoll and Winter, 1987). Thus, phenol was used as a reference for 4-CP. According to the Buswell equation, the stoichiometry of phenol degradation is as follows:

$$C_6H_6O + 4H_2O \longrightarrow 2.5CO_2 + 3.5CH_4$$

Although greater than 84% of the radioactivity from phenol-degrading cultures was recovered (Tables 13b and 14b), it was not possible to determine the stoichiometry because a large proportion of the radioactivity was still present in the solids and liquids. However, culture 4 exhibited a methane plateau for nearly two weeks, and even this culture still had 18% of the radioactivity in the solids and liquids. This culture was used to monitor methane production in cultures 1 to 3, which had produced the same amounts of gas as culture 4. Similarly, culture 8 was used to monitor methane production in cultures 5 to 7. HPLC analysis of culture 5, as a representative of the other cultures, showed the presence of a large amount of a compound with the same retention time as authentic acetate in solution (Figure 36), explaining the incomplete degradation. Thus, the methanogens involved in splitting acetate into carbon dioxide and methane were not present in enough numbers to perform this final step, or they were inactive.

Another problem with the phenol-degrading cultures was the large amounts of carbon dioxide produced in comparison to methane. Cultures 1 to 8 (Tables 13b and 14b) were prepared using 2-year-old radioactive phenol. The average methane to carbon dioxide ratio for these cultures was 0.62 ± 0.05. According to the stoichiometry, the molar ratio of methane to carbon dioxide should be 1.4. If the gases are corrected for solubility as shown in Appendix F, the molar ratio should be 3.7. Even though these cultures had not completely converted all of the acetate to gaseous products, there was some factor that was contributing to the high carbon dioxide production.

This problem lead to the analysis of the 2-year-old phenol stock solution. The non-acidified phenol was analyzed, and because the phenol was only 71% pure with an additional 5% acetate, it was concluded that the stock solution was impure, and decomposition of the phenol had taken place. The phenol stock was then acidified to determine the difference in radioactivity recovery in comparison to the non-acidified sample. A difference of nearly 40% in the radioactivity recovery meant that other volatile components were present that were volatilized at a pH of 1.

The use of a new batch of radioactive phenol did not make a difference in improving the results. Because such a large amount of the radioactivity stayed with the liquids and solids, it was not possible to determine conclusively that the methane to carbon dioxide ratio would be closer to that expected. Also, the culture appeared to be much slower at degrading phenol, again leading to the conclusion that the methane had plateaued. The cultures prepared with the new radioactive phenol were cultures 9 to 12, A1 to D1, and A2 to D2 (Tables 16b, 17, and 18).

The cultures containing radioactive phenol at two concentrations were prepared to determine whether carbon dioxide and methane production would be higher with a lower concentration of phenol because the cultures were not that active. That is, it may have been more difficult for the cultures to degrade a higher concentration of phenol. Again, there was not significant degradation for either of the two sets of cultures because so much of the radioactivity was still present in both the solids and liquids. While flushing cultures A1 to D1, some fluor entered the lines, accounting for some loss in radioactivity (Table 17). Cultures A2 to

D2, however, did not have this problem, yet still had similar percentages of carbon dioxide present (Table 18). Therefore, in cultures A1 to D1, the presence of fluor in the lines did not lead to significant losses. Knoll and Winter (1987) found that the methane to carbon dioxide ratio in experiments using radioactive phenol as the substrate was 1.4 as predicted (Appendix F). However, they were able to maintain a substrate concentration of 200 mg/L with their cultures.

The low methane production in all of the cultures containing radioactive phenol lead to the experiment observing methane production in cultures containing non-radioactive phenol. A comparison of methane production using a low and high phenol concentration was conducted because previous research regarding phenol degradation used phenol concentrations of approximately 300 mg/L. The low phenol concentration was used in radioactivity recovery experiments because the 4-CP concentrations were low, and it was thought that recoveries would be more comparable if both types of cultures contained low concentrations of substrate. Again, the methane plateau was used to determine the end of the experiment.

The methane production observed in cultures containing nearly 26 mg/L phenol was 0.81 mL ± 0.072 mL methane/mg phenol (Table 21), and this value was very close to the predicted values of 0.75 mL/mg phenol (Fedorak, 1984) and 0.83 mL/mg phenol based on Buswell's equation. However, the higher phenol concentrations had methane values that were much higher than predicted. One reason for this may have been caused by the use of the calibration curve for the higher concentrations of phenol. The calibration curve for the lower concentrations had been constructed from points that differed by approximately 7 mg/L, but the curve for the higher concentrations had been constructed from points that differed by 50 to 100 mg/L. Thus, there was more room for error, especially seen by the scatter in the last point taken.

10.8.2. ^{14}C-4-Chlorophenol-containing cultures

The stoichiometry of 4-CP biodegradation was important in characterizing the reaction. By recovering the radioactivity from cultures after 4-CP was completely degraded, it was presumed that most of the label would be present as carbon dioxide and methane.

Krumme and Boyd (1988) used the following formula to demonstrate the stoichiometry of the degradation of 4-CP:

$$C_6H_4OHCl + 4.5H_2O \longrightarrow 2.75CO_2 + 3.25CH_4 + HCl$$

This formula was chosen to demonstrate the assumption that dehalogenation proceeded via the production of HCl. This resulted in a methane to carbon dioxide ratio of 1.18. If the solubility of the gases is taken into account as shown in Appendix F, the molar ratio becomes 3.2. A problem with the stoichiometry in the above equation as well as that for phenol shown in section 10.8.1. was that the incorporation of carbon into the microorganisms was not taken into account. That is, 100% of the substrate would not be mineralized because some of the carbon would be needed for microbial growth. Kirsch and Sykes (1971) found that during anaerobic biodegradation, 10% to 20% of the substrate was converted to biomass. Sahm (1984) found that only 5% of the substrate was converted to biomass, whereas 95% was converted to gas.

In this study, the cultures containing radioactive 4-CP were not converting all of the substrate to gaseous products as evidenced by the large amounts of radioactivity in the solids and liquids. This was a reflection of the inoculum because all 4-CP-containing cultures were prepared from batch cultures that contained the same initial inoculum. Thus, there were low bacterial numbers present, primarily the acetate-degraders because large amounts of acetate were found in the supernatant after it was presumed that degradation was complete. The highest percentage of carbon dioxide produced in the radioactive cultures was 43% (Table 9), and the highest amount of methane was 31.0% (Table 12b). Even these values leave nearly 30% of the label in the solids and liquids. Cultures M, N, and P were producing low amounts of methane (Table 12b). Dolfing and Tiedje (1986) noted that lower amounts of methane were recovered with substrates that were chlorinated. They believed that this was caused by the use of hydrogen as a co-substrate in which hydrogen was consumed resulting in less methane production. This could be an explanation for the low methane production in this project. However, further studies would be needed to conclude that this was the case. Cultures M, N, and P were not analyzed further to determine the proportion of radioactivity present in the solids and liquids because of the extremely long period of time taken by these cultures to degrade the substrate. It was thought that these cultures had either lost some of the bacterial population present or had become inactive. This could be assumed from the extremely low 4-CP degradation rate of 0.3 mg/L/day for culture Q (Figure 34), the culture initially used to monitor cultures M, N, and P. Another reason for the low and varying rates for the 4-CP cultures may be because the 4-CP could not be used as the primary carbon and energy source. That is, the cultures contained only 4-CP as the major carbon source, but if another substrate is provided that could serve as the carbon source, this may facilitate 4-CP degradation. A possible carbon source is phenol because it has been shown in this research to degrade quickly in fresh sludge.

Krumme and Boyd (1988) used labelled 4-CP and observed only 19.4% radioactive carbon dioxide. However, 24.9% methane was based on a calculation based on assumed stoichiometry, rather than an analysis. This resulted in a methane to carbon dioxide ratio of 1.28. Similarly, Mikesell and Boyd (1986) calculated percent methane from a radioactive pentaCP label based on the assumption that phenol was an intermediate, and determined a methane (38.3%) to carbon dioxide (27.4%) ratio of 1.4. Boyd and Shelton (1984) found that 50% of both radioactive carbon dioxide and methane was produced with radioactive 4-CP as a label in an acclimated 4-CP culture. In this case, both gases were observed, resulting in a ratio of 1 for methane to carbon dioxide, which was close to the theoretical ratio of 1.18 without correcting for solubility (Appendix F).

11. Conclusions

This research primarily focused on the identification of intermediates during 4-CP degradation, as well as the stoichiometry involved.

The sludge proved to be a problem as an inoculum. Active bacterial numbers were probably inadequate because of the long lag periods prior to substrate degradation.

Differences in degradation rates between batch cultures further complicate matters. That is, it was difficult to obtain a homogeneous bacterial population from a complex inoculum containing adequate levels of active bacteria with appropriate proportions of the types required to carry out methanogenesis. Because the consortium contains several types of interdependent organisms, an effective, homogeneous population is difficult to obtain. Ideally, a continuous digestor enriched with 4-CP will ensure both a homogeneous and active bacterial population. Enrichment for a long period of time is necessary so that the microbes have had several feedings with the substrate to keep the enzymes necessary to degrade 4-CP actively. A few feedings prior to experimentation may not ensure very active populations. The presence of the acetate intermediate suggests that the methanogen activity was a problem throughout these experiments.

In the cultures containing radioactive 4-CP, the substrate was adsorbed to the solids. Based on Dobbs et al. (1989), the calculated logs Kp' of the adsorption coefficients for 2,4-diCP and 4-CP were 3.0 and 2.5, respectively. Based on the work in this project, the observed value of log Kp' for 2,4-diCP was 2.2.

Dechlorination was proven for 2-CP and 2,4-diCP with the resultant production of phenol and 4-CP, respectively. However, although 4-CP was degraded, the production of phenol was not observed. Therefore, it is not certain whether the chlorine is removed from 4-CP before or after the aromatic ring is cleaved.

The radioactivity recovery method utilizing the Swinex filters (third radioactivity recovery procedure) proved to be the best method in terms of minimizing losses of radioactivity when analyzing the solids and the supernatant. However, the stoichiometry of phenol and 4-CP degradation could not be determined because of the high amounts of radioactivity in the liquids and solids of both the phenol-containing and 4-CP-containing cultures.

Degradation rates of 4-CP varied widely between cultures taken from the same initial batch culture. This finding further illustrated the difficulty in obtaining a homogeneous bacterial population. Cultures in which the concentration of 4-CP was closely followed so that the second feeding was given close to the time the first substrate was depleted exhibited the highest degradation rates. Therefore, close monitoring of the substrate will likely help to obtain an active population.

Mineralization of 4-CP was proven by detecting the radioactive carbon dioxide and methane produced. However, the only extracellular intermediate identified had the same retention time as authentic acetate. This does, however, support the ring cleavage pathway. If phenol was present, it was present in non-detectable amounts, or may have been degraded as rapidly as it was produced also making detection difficult.

12. Recommendations for further research

1) A continuous digestor instead of batch cultures would have a higher degradation rate once the culture is acclimated, assuming the consortium contains adequate numbers of all organisms.

2) Also, with a continuous digestor, the 4-CP concentration can gradually be increased to higher levels, so that it may be easier to detect intermediates.

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14. Appendix A: Data for figures

Table A1. Data for Figure 7 (Adsorption of 2,4-Dichlorophenol to Sterile and Non-sterile Sludge I)

Time (Hours)	2,4-Dichlorophenol (mg/L) Sterile cultures	Non-sterile cultures	Sterile water
0	10.4	8.30	14.7
0.083	10.2	7.40	-
0.250	9.80	7.70	-
0.500	9.80	8.70	15.7
0.750	10.1	8.80	-
1.00	10.1	8.90	14.7
2.00	9.90	9.50	15.4
3.00	9.50	9.70	-
3.33	-	-	15.1
3.72	-	-	15.3
4.00	9.50	9.00	-
5.00	9.00	9.80	-
5.33	-	-	14.7
5.72	-	-	16.4
6.00	9.80	9.90	-
7.00	9.30	10.0	-
7.33	-	-	18.8
7.72	-	-	16.6

- = Not measured

Table A2. Data for Figure 8 (Adsorption of 2,4-Dichlorophenol to Sterile and Non-sterile Sludge II)

Time (Hours)	2,4-Dichlorophenol (mg/L) Sterile cultures	Non-sterile cultures	Sterile water
0	19.1	19.1	16.7
1.00	16.5	17.6	11.7
2.00	17.5	16.9	12.3
3.00	15.4	16.0	11.0
4.00	15.5	14.8	12.8
5.00	16.1	12.8	14.5
23.5	15.1	-	14.7
24.5	15.9	-	18.8
26.0	-	19.6	16.4
27.0	-	18.2	16.6

- = Not measured

Table A3. Data for Figure 9 (Dechlorination of 2-Chlorophenol by an Established Digestor)

Days incubation	2-CP (mg/L)
0	27.0
1	23.9
2	20.7
5	11.2
7	6.05
9	1.53

Table A4. Data for Figure 10 (Dechlorination of 2-Chlorophenol Using Different Inocula)

Days incubation	2-Chlorophenol (mg/L) Fresh sludge	Phenol 3	Phenol 4	2-CP Digestor
0	26.8	27.8	27.6	33.9
1	30.9	31.9	32.4	43.5
2	27.1	18.5	31.7	40.8
3	24.7	21.1	33.4	44.6
4	14.2	19.9	29.6	42.2
5	1.76	10.4	24.1	31.0
6	0	17.7	27.8	37.2

Table A5. Data for Figure 11 (Dechlorination of 2-Chlorophenol by Diluted Sludge Cultures)

	2-Chlorophenol (mg/L)			
Days incubation	1/8	1/4	1/2	Undiluted
0	25.0	25.0	25.0	25.0
4	22.9	22.9	21.7	19.0
7	23.4	17.4	7.40	1.95
11	21.4	12.6	0.45	0
14	18.5	2.87	0	-
18	14.6	-	-	-
21	9.30	-	-	-
25	3.49	-	-	-
28	0	-	-	-
32	-	-	-	-
39	-	-	-	-

- = Not measured

Table A6. Data for Figure 12 (Phenol Production from Diluted Sludge Cultures)

	Phenol (mg/L)			
Days incubation	1/8	1/4	1/2	Undiluted
4	0	0.34	2.13	4.37
7	0.60	2.47	9.95	13.6
11	1.86	8.88	12.3	6.68
14	3.64	14.0	7.18	0
18	6.65	12.3	0	-
21	10.8	6.48	-	-
25	13.1	0	-	-
28	14.0	-	-	-
32	6.37	-	-	-
39	0	-	-	-

- = Not measured

Table A7. Data for Figure 13 (Methane Production from Diluted Sludge Cultures Containing 2-Chlorophenol)

	Methane production (mL)			
Days incubation	1/8	1/4	1/2	Undiluted
4	0.09	0.44	1.05	1.82
7	0.25	0.53	1.23	2.16
11	0.29	0.52	1.25	2.35
14	0.16	0.53	1.17	2.38
18	0.21	0.60	1.79	2.40
21	0.27	0.86	1.90	2.50
25	0.27	1.17	2.06	2.72
28	0.34	1.39	2.09	-
32	0.51	1.39	2.23	-
35	0.69	1.52	2.52	3.00
39	0.88	1.21	1.89	2.72
42	-	1.50	-	-
46	0.91	0.91	1.57	2.17
49	0.87	0.80	1.79	2.70
53	0.79	0.77	1.32	1.84
56	0.98	0.95	1.59	2.66

- = Not measured

Table A8. Data for Figure 14 (Analysis of 4-CP A Supernatant by HPLC and Fractionation I)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0.25	26	0.74
2	0.99	27	0.25
3	0.37	28	2.34
4	0.25	29	14.9
5	0.49	30	13.7
6	0.74	31	9.73
7	0.62	32	5.79
8	0	33	2.46
9	1.48	34	1.97
10	0.37	35	1.11
11	0.37	36	1.35
12	0.12	37	0.12
13	0	38	0.74
14	0	39	0.74
15	1.85	40	0.49
16	0.25	41	0
17	1.48	42	0
18	0.12	43	0.74
19	0.12	44	1.11
20	0.12	45	0
21	0.86	46	0
22	0	47	0.86
23	0.25	48	0.86
24	0	49	0.25
25	0.25	50	0

Table A9. Data for Figure 15 (Analysis of 4-CP A Supernatant by HPLC and Fractionation II)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	1.16	26	1.30
2	1.30	27	1.30
3	2.02	28	4.77
4	1.16	29	15.9
5	1.30	30	17.5
6	1.45	31	8.96
7	0.43	32	6.65
8	0.43	33	3.32
9	0.72	34	2.89
10	1.30	35	1.30
11	1.01	36	1.88
12	1.01	37	0.87
13	0.43	38	1.30
14	1.01	39	0.87
15	0.43	40	0.87
16	1.88	41	1.45
17	0.58	42	0.29
18	2.02	43	0.14
19	0	44	1.45
20	1.01	45	0.58
21	1.30	46	1.16
22	1.88	47	0
23	1.01	48	0.72
24	0.29	49	0.87
25	0	50	0.43

Table A10. Data for Figure 16 (Analysis of Standard 4-CP by HPLC and Fractionation)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0.02	26	0.06
2	0.04	27	1.94
3	0.05	28	24.3
4	0.05	29	27.3
5	0.05	30	18.5
6	0.03	31	11.4
7	0.03	32	6.25
8	0.05	33	3.14
9	0.02	34	1.33
10	0.03	35	0.55
11	0.02	36	0.22
12	0.03	37	0.14
13	0.06	38	0.07
14	0.08	39	0.06
15	0.08	40	0.05
16	0.11	41	0.08
17	0.08	42	0.03
18	0.07	43	0
19	0.09	44	0.07
20	0.12	45	0.07
21	0.05	46	0.04
22	0.12	47	0.08
23	0.09	48	0.03
24	0.04	49	0.05
25	0.05	50	0.09

Table A11. Data for Figure 17 (Analysis of 4-CP A Supernatant Plus Standard 4-CP by HPLC and Fractionation I)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0.23	26	0.20
2	0.02	27	0.05
3	0.05	28	4.51
4	0.20	29	21.2
5	0.23	30	23.6
6	0.07	31	16.9
7	0.16	32	10.6
8	0.05	33	6.38
9	0.20	34	3.21
10	0.02	35	1.80
11	0.18	36	0.52
12	0.02	37	0.11
13	0.07	38	0.18
14	0.23	39	0.07
15	0.16	40	0
16	0.23	41	0.11
17	0.39	42	0.07
18	0.02	43	0
19	0.27	44	0.14
20	0.27	45	0.05
21	0.05	46	0.07
22	0	47	0.02
23	0.18	48	0.09
24	0.02	49	0.05
25	0.05	50	0.27

Table A12. Data for Figure 18 (Analysis of 4-CP A Supernatant by HPLC and Fractionation III)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0.98	31	0
2	0	32	0
3	0	33	0.65
4	0	34	0.65
5	0.65	35	0
6	3.92	36	0
7	6.54	37	0
8	0.65	38	0.33
9	0	39	0
10	0.65	40	1.96
11	0.65	41	0
12	0	42	0
13	0	43	0
14	0	44	0
15	2.61	45	0.33
16	0	46	0
17	0.98	47	0
18	1.63	48	0
19	0.98	49	0
20	0.65	50	0
21	0.98	51	0
22	0	52	0.98
23	0	53	0
24	0	54	0.33
25	1.96	55	0
26	0	56	0
27	0	57	0.33
28	2.29	58	0
29	0	59	0
30	1.31	60	0

Table A13. Data for Figure 19 (Analysis of 4-CP A Supernatant Plus Standard 4-CP by HPLC and Fractionation II)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0	21	0
2	0	22	0.05
3	0.12	23	0
4	0.42	24	0
5	0.35	25	0.07
6	40.2	26	0.07
7	48.4	27	0.02
8	1.41	28	0
9	0.16	29	0
10	0	30	0.21
11	0.26	31	0
12	0.12	32	0
13	0.24	33	0
14	0.31	34	0.14
15	0.12	35	0.02
16	0.02	36	0
17	0.07	37	0.02
18	0	38	0
19	0	39	0
20	0		

Table A14. Data for Figure 20 (Degradation of 4-Chlorophenol by Culture F)

Days incubation	4-CP (mg/L)
6	33.1
8	28.1
14	18.1
15	18.1
17	9.71
18	9.45
19	9.23
20	7.07
21	4.68
22	4.31
23	2.21
25	33.1
26	32.5
27	27.4
28	29.1
29	25.3
30	24.1
31	22.2
32	20.8
33	20.8
34	16.9
35	15.5
36	10.9
37	9.55
42	4.94

Table A15. Data for Figure 21 (Degradation of 4-Chlorophenol by Culture G)

Days incubation	4-CP (mg/L)
0	39.5
1	35.8
2	35.8
5	33.1
6	28.3
8	24.9
9	24.0
10	23.5
11	21.7
12	21.4
13	20.4
14	17.4
16	11.9
17	9.76
18	6.83
19	2.83
20	3.65
21	0.87
21	41.4
24	28.6
28	14.6
29	9.06

Table A16. Data for Figure 22 (Degradation of 4-Chlorophenol by Culture H)

Days incubation	4-CP (mg/L)
0	37.8
1	39.4
2	37.3
5	33.5
6	31.4
8	28.1
9	27.4
10	29.1
11	25.8
12	29.3
13	29.0
14	26.9
16	24.9
17	25.1
18	21.7
19	23.8
20	20.0
21	19.4
22	17.7
23	16.2
24	17.8
25	15.4
26	13.6
27	10.5
28	9.55
29	7.76
30	4.06
31	3.60
33	3.93
34	35.2
35	28.3

Table A17. Data for Figure 23 (Degradation of 4-Chlorophenol by Culture J)

Days incubation	4-CP (mg/L)
0	35.2
1	38.9
2	34.0
5	31.9
6	33.1
8	26.6
9	27.7
10	29.1
11	27.1
12	26.4
13	27.0
14	23.9
16	19.3
17	22.3
18	17.3
19	16.9
20	15.0
21	11.9
22	9.67
23	8.79
24	6.71
25	5.97
26	5.42
26	34.5
28	25.0
30	20.3

Table A18. Data for Figure 24 (Analysis of Culture G Supernatant by HPLC and Fractionation: Solvent I)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0	31	0
2	0	32	0
3	0.18	33	0
4	7.22	34	0
5	5.25	35	0
6	80.1	36	0
7	7.97	37	0
8	0.12	38	0
9	0	39	0
10	0.04	40	0
11	0	41	0.08
12	0	42	0
13	0.02	43	0
14	0	44	0.04
15	0	45	0.02
16	0	46	0
17	0	47	0.10
18	0	48	0
19	0.04	49	0.10
20	0	50	0
21	0	51	0
22	0	52	0
23	0	53	0
24	0	54	0
25	0	55	0
26	0	56	0
27	0	57	0
28	0.02	58	0
29	0	59	0
30	0	60	0

Table A19. Data for Figure 25 (Analysis of Culture G Supernatant by HPLC and Fractionation: Solvent II)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0.17	36	0.13
2	0	37	0.10
3	0	38	0.20
4	0.34	39	0.13
5	0.67	40	0.30
6	4.32	41	0.10
7	2.65	42	0.34
8	0.77	43	0.10
9	0.54	44	0.13
10	0.27	45	0.03
11	0.70	46	0
12	0.30	47	0
13	0.27	48	0.20
14	0.17	49	0.13
15	0.34	50	0
16	1.37	51	0.23
17	47.3	52	0.23
18	34.9	53	0.44
19	5.46	54	0.10
20	0.34	55	0.17
21	0.30	56	0.17
22	0.10	57	0.03
23	0.30	58	0.30
24	0.34	59	0.13
25	0.30	60	0.20
26	0.20	61	0.20
27	0.37	62	0.10
28	0	63	0.23
29	0.13	64	0.20
30	0.10	65	0.13
31	0.07	66	0
32	0.20	67	0.20
33	0.23	68	0.23
34	0.34	69	0.13
35	0.13	70	0.30

Table A20. Data for Figure 26 (Analysis of Standard Acetate by HPLC and Fractionation: Solvent II)

Fraction	% DPM Injected
1	1.45
2	0.62
3	2.07
4	0
5	4.13
6	66.7
7	21.1
8	2.27
9	0.62
10	1.86
11	0.83
12	1.65
13	2.07
14	1.24
15	0
16	0.62
17	0.21
18	1.45
19	1.03
20	1.24

Table A21. Data for Figure 27 (Analysis of Culture G Supernatant by HPLC and Fractionation: Solvent III)

Fraction	%DPM Injected	Fraction	%DPM Injected
1	0.20	21	36.8
2	0.56	22	0.87
3	6.11	23	0.15
4	0.15	24	0.05
5	0.20	25	0.15
6	0.51	26	0.10
7	0.25	27	0
8	0.56	28	0
9	0	29	0.15
10	0.20	30	0
11	0.25	31	0
12	0	32	0.10
13	0	33	0
14	0	34	0
15	0.10	35	0.05
16	0	36	0
17	0.10	37	0
18	0.25	38	0.20
19	3.21	39	0
20	49.2	40	0.20

Table A22. Data for Figure 28 (Analysis of Standard Acetate by HPLC and Fractionation: Solvent III)

Fraction	%DPM Injected
1	1.38
2	0.75
3	0.21
4	6.82
5	78.6
6	6.39
7	0.96
8	1.06
9	1.28
10	0.53

Table A23. Data for Figure 29 (Analysis of Culture H Supernatant by HPLC and Fractionation: Solvent II)

Fraction	% DPM Injected
1	0.03
2	0.05
3	0.04
4	0.08
5	0.15
6	1.39
7	0.63
8	0.12
9	0.15
10	0.07
11	0.24
12	0.15
13	0.11
14	0.03
15	0.07
16	0.70
17	45.6
18	43.9
19	6.39
20	0.49
21	0.12
22	0.10
23	0.10
24	0.03
25	0.03
26	0.08
27	0.18
28	0.01
29	0
30	0.05
31	0.04
32	0.06
33	0.10
34	0.05
35	0
36	0.07
37	0
38	0.04
39	0.04
40	0.08
41	0.01
42	0.10
43	0.01
44	0.08
45	0.01
46	0.05
47	0.05
48	0.01
49	0
50	0.07
51	0
52	0.03
53	0.06
54	0.05
55	0.04
56	0.08
57	0.03
58	0.10
59	0
60	0.09
61	0.10
62	0.05
63	0.06
64	0.10
65	0.05
66	0.06
67	0.08
68	0.05
69	0
70	0.04

Table A24. Data for Figure 30 (Analysis of Culture H Supernatant by HPLC and Fractionation: Solvent III)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0	21	42.6
2	0.10	22	1.42
3	1.84	23	0.08
4	0.09	24	0.08
5	0.09	25	0.08
6	0	26	0.01
7	0.10	27	0
8	0	28	0
9	0	29	0.05
10	0	30	0.01
11	0.10	31	0
12	0.08	32	0.01
13	0	33	0
14	0	34	0
15	0	35	0
16	0.01	36	0
17	0	37	0.14
18	0.03	38	0.06
19	2.72	39	0
20	49.6	40	0.10

Table A25. Data for Figure 31 (Analysis of Culture J Supernatant by HPLC and Fractionation: Solvent I)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0	31	0
2	0	32	0
3	0	33	0
4	3.22	34	0
5	3.50	35	0
6	84.0	36	0
7	8.58	37	0
8	0.05	38	0
9	0.01	39	0.04
10	0	40	0
11	0	41	0
12	0	42	0
13	0	43	0
14	0	44	0
15	0	45	0
16	0	46	0
17	0	47	0
18	0	48	0.01
19	0	49	0
20	0	50	0
21	0	51	0
22	0	52	0
23	0	53	0
24	0	54	0
25	0	55	0
26	0	56	0
27	0	57	0
28	0	58	0
29	0.02	59	0.01
30	0	60	0

Table A26. Data for Figure 32 (Analysis of Culture J Supernatant by HPLC and Fractionation: Solvent II)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0	36	0.04
2	0	37	0.10
3	0.09	38	0.06
4	0.03	39	0.04
5	0.30	40	0
6	3.29	41	0
7	1.47	42	0.09
8	0.35	43	0
9	0.17	44	0
10	0.12	45	0.09
11	0.23	46	0.07
12	0.19	47	0.16
13	0.16	48	0.06
14	0.16	49	0.09
15	0.07	50	0.19
16	1.57	51	0.13
17	52.6	52	0.09
18	35.8	53	0.09
19	4.42	54	0.09
20	0.26	55	0.13
21	0.04	56	0.14
22	0.09	57	0.09
23	0.17	58	0.14
24	0.04	59	0
25	0.09	60	0.07
26	0.06	61	0.10
27	0.01	62	0.10
28	0.16	63	0.16
29	0.16	64	0.10
30	0.12	65	0.03
31	0.12	66	0.06
32	0.01	67	0
33	0.09	68	0
34	0.13	69	0.04
35	0.04	70	0.19

Table A27. Data for Figure 33 (Analysis of Culture J Supernatant by HPLC and Fractionation: Solvent III)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0.02	21	31.7
2	0	22	0.60
3	4.49	23	0.06
4	0	24	0.19
5	0.02	25	0
6	0.09	26	0.13
7	0	27	0
8	0	28	0
9	0.09	29	0.02
10	0	30	0
11	0.06	31	0.15
12	0	32	0.02
13	0	33	0
14	0	34	0.09
15	0	35	0
16	0	36	0
17	0.06	37	0.02
18	0.09	38	0
19	4.00	39	0.04
20	56.5	40	0

Table A28. Data for Figure 34 (Degradation of 4-Chlorophenol by Culture Q)

Days incubation	4-CP (mg/L)
0	31.1
4	29.3
7	26.2
10	24.9
12	23.4
18	22.4
21	26.5
24	23.2
27	21.6
32	22.3
40	20.1
53	17.1
59	15.4
74	9.60
87	0.64

Table A29. Data for Figure 35 (Degradation of Phenol by Culture 4)

Days incubation	Phenol (mg/L)
0	22.7
1	21.6
2	21.5
3	22.3
6	21.0
7	21.4
8	19.6
9	19.1
14	4.69

Table A30. Data for Figure 36 (Analysis of Culture 5 Supernatant by HPLC and Fractionation: Solvent III)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0	21	1.48
2	3.11	22	0.78
3	27.6	23	0.31
4	10.3	24	0.47
5	5.51	25	0.16
6	4.89	26	0.31
7	3.49	27	0.62
8	1.40	28	0.78
9	1.79	29	0.70
10	2.17	30	0
11	0.78	31	0.47
12	1.01	32	0.70
13	0.93	33	0.31
14	1.79	34	0.23
15	1.48	35	0
16	1.32	36	0.62
17	0.78	37	0.39
18	1.24	38	0.16
19	0.47	39	0.16
20	0.93	40	0

Table A31. Data for Figure 37 (HPLC Analysis of Acidified Phenol Stock Solution by HPLC and Fractionation: Solvent III)

Fraction	% DPM Injected	Fraction	% DPM Injected
1	0	21	0
2	0	22	0
3	0	23	0
4	0.84	24	0.09
5	1.43	25	0
6	0.40	26	0
7	0.28	27	0.09
8	0.05	28	0
9	0.23	29	0
10	0.07	30	0
11	3.51	31	0
12	18.1	32	0
13	2.36	33	0
14	0.05	34	0
15	0.09	35	0
16	0	36	0
17	0.05	37	0.05
18	0.14	38	0.16
19	0	39	0
20	0	40	0

Table A32. Data for Figure 38 (Analysis of Non-acidified Phenol Stock Solution by HPLC and Fractionation: Solvent III)

Fraction	% DPM Injected
1	0
2	2.90
3	4.05
4	1.09
5	0.38
6	25.9
7	43.0
8	0.71
9	0.66
10	0.44
11	0.27
12	0.22
13	0
14	0.71
15	0.16
16	0
17	0.16
18	0.33
19	0
20	0.33

Table A33. Data for Figure 39 (Analysis of Non-acidified 4-CP Stock Solution by HPLC and Fractionation: Solvent III)

Fraction	% DPM Injected
1	0
2	0.15
3	0.65
4	0
5	0
6	0
7	0.17
8	0.07
9	0.04
10	0
11	0
12	0.17
13	0.13
14	0
15	0.13
16	0.07
17	0.02
18	0.06
19	0.69
20	17.1
21	63.6
22	13.5
23	0.45
24	0.20
25	0
26	0
27	0
28	0.07
29	0.02
30	0
31	0.06
32	0
33	0.04
34	0.02
35	0

Table A34. Data for Figure 40 (Calibration Curves for Low and High Phenol Concentrations)

Area	Phenol (mg/L)
0.029	5.30
0.038	5.30
0.038	5.30
0.080	12.7
0.082	12.7
0.133	19.4
0.143	19.4
0.215	26.8
0.206	26.8
0.221	33.2
0.252	33.2
1.69	156
1.61	156
2.41	201
2.18	201
3.58	314
3.46	314
3.55	314
4.76	352
4.42	352

Table A35. Data for Figure 41 (Methane Plateaus for High and Low Phenol Concentrations)

	Volume methane above control (mL)	
Days incubation	High phenol	Low phenol
0	0	0
2	0.170	2.22
4	0.480	2.66
7	0.800	2.45
9	1.13	2.58
11	1.67	2.65
14	-	2.54
16	3.36	-
18	3.22	-
21	3.11	-

- = Not measured

15. Appendix B: Phenol production from 2-chlorophenol dechlorination

The calculation shown here was to explain the value of 18.3 mg/L phenol expected if 25 mg/L 2-CP was completely dechlorinated (section 9.7.2.2.1. in Results).

Because 2-CP has been shown to be dechlorinated to phenol, it is possible to predict the concentration of phenol expected based on an initial 2-CP concentration using the following stoichiometry:

$$H_2 + C_6H_5ClO \longrightarrow C_6H_6O + H^+ + Cl^-$$

A concentration of 25 mg/L 2-CP is equivalent to 0.195 mM. Based on the stoichiometry of the above equation, a maximum of 0.195 mM or 18.3 mg/L phenol should be produced.

16. Appendix C: Methane production from phenol biodegradation

When determining methane production based on the initial concentration of phenol present, two relations were used. The first was based on Buswell's equation (Buswell and Mueller, 1952) which results in the following equation for the production of methane and carbon dioxide from phenol degradation:

$$C_6H_6O + 4H_2O \longrightarrow 2.5CO_2 + 3.5CH_4$$

In this case, 1 mg of phenol produces 0.83 mL methane.

The second relation was based on Fedorak (1984) in which 1 mg phenol was found to produce 0.75 mL of methane.

Therefore, for 18.3 mg/L phenol:

a) By the first method,

18.3 mg phenol x 0.83 mL methane/mg phenol

= 15.2 mL methane produced

b) By the second method,

18.3 mg phenol x 0.75 mL methane/mg phenol

= 13.7 mL methane produced.

17. Appendix D: Determination of the degree of adsorption of 2,4-dichlorophenol to sludge

Calculations are based on those used by Woods (1984) to explain the results of the experiment, Sludge adsorption I (section 9.5.1. in Results).

Woods (1984) used the following relationship to determine the degree to which chlorophenols adsorbed to anaerobic reactor solids:

$$(P_S) = K_F(P_L)^{1/n}$$

where:

(P_S) = total concentration of the chlorophenol in the solids phase, µM/L

(P_L) = total concentration of the chlorophenol in the liquid phase, µM/L

K_F = Freundlich constant, L/g VSS

$1/n$ = exponent.

Because n can range from 0.9 to 1.3, a value of 1.0 will be used in which case the above relationship becomes an equation characterizing the distribution coefficient. Therefore, the concentration of chlorophenols in the liquid and solid phases can be linearly related.

Thus, with n = 1.0, the equation becomes:

$$K_S = \frac{P_S}{P_L}$$

where K_S = the distribution coefficient, L/g VSS.

Because the results for Sludge adsorption II (Figure 8) were not conclusive, the VSS for the cultures (Table 8) will be used in the calculations for Sludge adsorption I (Figure 7). Because the inoculum and culture preparation were the same for both adsorption experiments, it was presumable that the VSS for the cultures in Sludge adsorption I would have been similar to those for Sludge adsorption II.

The average concentration of 2,4-diCP in sterile water was 15.7 mg/L (based on the data in Table A1 in Appendix A). Assuming there may have been a problem with the addition of the 2,4-diCP stock solution to the cultures and sterile water, the 15.7 mg/L 2,4-diCP was the actual concentration initially added to the cultures and sterile water, instead of 25 mg/L.

The concentration of 2,4-diCP in the sterile culture at the end of the experiment was 9.3 mg/L (based on the data in Table A1 in Appendix A).

The VSS for the sterile cultures was 4.03 x 10^3 mg/L (Table 8).

Therefore,

$$P_S = \frac{(15.7\ \text{mg/L} - 9.3\ \text{mg/L})(\text{Volume})}{(4.03\ \text{g/L VSS})\ (\text{Volume})}$$

$$= 1.59\ \text{mg 2,4-diCP/g VSS}$$

$$P_L = 9.3\ \text{mg 2,4-diCP/L}$$

$$K_S = \frac{P_S}{P_L}$$

$$= \frac{1.59\ \text{mg 2,4-diCP/g VSS}}{9.3\ \text{mg 2,4-diCP/L}}$$

$$= 0.171\ \text{L/g VSS}$$

Woods (1984) found that the K_S for 2,4-diCP ranged from 0.20 to 0.39 L/g VSS. The calculated value of 0.171 L/g VSS was slightly below this range.

K_S = 171 L/kg VSS   based on 1 L = 1 kg.

$$Kp' = 1000K_S$$
$$= 171$$

$\log Kp' = 2.2$

Dobbs et al. (1989) used calculated adsorption coefficients (Kp') for toxic organic compounds to determine an equation correlating the Kp' to the K_{ow}. The calculated log Kp' was based on adsorption to wastewater solids. The equation derived from the correlations was as follows:

$$\log Kp' = 0.58 \log K_{ow} + 1.14.$$

Based on this equation, the log Kp' for 2,4-diCP is 3.0, compared to the measured value of 2.2 for this work.

To determine the expected loss of 2,4-diCP from solution, the K_S value of 0.3 L/g VSS will be used (0.3 L/g VSS is in the center of the range of 0.20 to 0.39 L/g VSS given by Woods, 1984).

$$K_S = 0.3 \text{ L/g VSS}$$
$$P_L = 9.3 \text{ mg/L}$$
$$P_S = (P_L)(K_S)$$
$$= (9.3 \text{ mg/L})(0.3 \text{ L/g VSS})$$
$$= 2.79 \text{ mg 2,4-diCP/g VSS}$$

The volume in the serum bottle was 50 mL,

50 mL x 4030 mg VSS/L

= 202 mg VSS

= 0.202 g VSS

Adsorbed 2,4-diCP = (2.79 mg 2,4-diCP/g VSS)(0.202 g VSS)

= 0.564 mg

Expected loss from solution = 0.564 mg/50 mL

= 11.3 mg/L

18. Appendix E: Use of Henry's Law to determine the volatility of 2,4-dichlorophenol in distilled water

Volume of serum bottle = 158 mL

V_g = volume of gas phase = 108 mL

V_l = volume of liquid phase = 50 mL

X_g = moles of 2,4-diCP in gas phase

X_l = moles of 2,4-diCP in liquid phase

25 mg/L 2,4-diCP in 50 mL = 1.25 mg

Molecular weight of 2,4-diCP = 164.01 g/mole

1.25 mg 2,4-diCP + 164.01 mg/mmole

= 7.6×10^{-6} moles

$$H = \frac{P_{VP}}{S} = 6.66 \times 10^{-6} \text{ atm·m}^3\text{/mol}$$

where:

P_{VP} = partial pressure of 2,4-diCP in gas phase, atm

S = concentration of 2,4-diCP in liquid phase, moles/m^3.

$$\frac{P_{VP}}{P_T} = \frac{X_g}{X_T}$$

where:

P_T = total gas pressure

X_T = total moles of gas.

P_T = 1 atm

1 mole @ 0°C $= 22.4$ L

@ 37°C $= \frac{310}{273} \times 22.4$ L

$= 25.4$ L

$$X_T = \frac{108 \text{ mL}}{25.4 \text{ L/mole}}$$

$$= 4.3 \times 10^{-3} \text{ moles}$$

$$H = \frac{P_{VP}}{S}$$

$$= \frac{(X_g/X_T)(P_T)}{X_l/\text{Volume}}$$

$$H = \frac{(X_g/4.3 \times 10^{-3} \text{ moles})(1 \text{ atm})}{X_l/5 \times 10^{-5} \text{ m}^3} = 6.66 \times 10^{-6} \text{ atm}\cdot\text{m}^3/\text{mol}$$

$$\frac{X_g}{X_l} = 5.7 \times 10^{-4}$$

Therefore, the amount of 2,4-diCP in the gas phase is negligible.

19. Appendix F: Theoretical percent methane and carbon dioxide production from phenol or 4-chlorophenol degradation

a) Phenol degradation,

Phenol stoichiometry:

$$C_6H_6O + 4H_2O \longrightarrow 2.5CO_2 + 3.5CH_4$$

Ratio	1	:	1.4		
	~42%	+	~58%		
Solubility coefficient in headspace	x 0.35		x 0.95		
	~14.7%	+	~55%	=	~70%
Ratio in headspace	1	:	3.7		

b) 4-CP degradation,

4-CP stoichiometry:

$$C_6H_4OHCl + 4.5H_2O \longrightarrow 2.75CO_2 + 3.25CH_4 + HCl$$

Ratio	1	:	1.18		
	~46%	+	~54%		
Solubility coefficient in headspace	x 0.35		x 0.95		
	~16%	+	~51%	=	~67%
Ratio in headspace	1	:	3.2		

Note: All solubility coefficients used were based on 35°C, although the incubation temperature was 37°C.

20. Appendix G: Duncans multiple range test

Calculations are based on Walpole and Myers (1989).

The example calculation uses the values for the 4-CP dilution experiment in section 9.7.1. of the Results section.

Values are as follows: (4 samples with 3 observations each)

	Sample 1	Sample 2	Sample 3	Sample 4
	24.6	23.6	21.0	23.2
	24.2	24.2	24.7	22.9
	24.3	25.6	23.8	23.9
$\overline{x}$	24.37	24.47	23.17	23.33

One Way Analysis of Variance Table

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Computed f
Treatment	4.14	3	2.07	1.63
Error	10.17	8	1.27	
Total	14.31	11		

Testing the hypothesis

$Ho: \mu_1 = \mu_2 = \mu_3 = \mu_4$

Against

Ha: At least two of the means are not equal

Critical region: $v_1 = 3$, $v_2 = 8$ degrees of freedom

$f > 4.07$ (5% confidence interval) from the F distribution

Since 1.63 is not greater than 4.07, do not reject Ho. Therefore, the concentrations (the means) are not significantly ($p < 0.05$) different.